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CHARACTERIZATION OF ENZYMES INVOLVED IN STORAGE PROTEIN HYROLYSIS IN CASTOR BEAN ENDOSPERM

BY

TITO MIGABO

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE

IN

PLANT MOLECULAR BIOLOGY

DEPARTMENT OF BIOLOGICAL SCIENCES

Edmonton, Alberta
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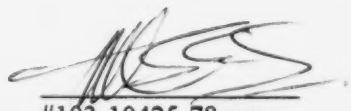

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The undersigned certify that they have read, and recommend to Faculty of Graduate Studies and Research for acceptance, a thesis entitled CHARACTERIZATION OF ENZYMES INVOLVED IN STORAGE PROTEIN HYDROLYSIS IN CASTOR BEAN ENDOSPERM submitted by Tito Migabo in partial fulillment of the requirements for the degree of MASTER OF SCIENCE in PLANT MOLECULAR BIOLOGY.

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Date: August 29, 1995

ABSTRACT

The crystalloid protein complex, making up 80 % of the total protein reserve of castor bean (Ricinus communis L. cv. Hale) seeds, is insoluble in aqueous buffers. During and following germination, this protein complex is hydrolyzed to soluble peptides and amino acids. The latter are used as a nitrogen source to support seedling development. Enzymes involved in the hydrolysis of the crystalloid protein complex were assayed using [^{3}H]-leucine and [^{35}S]-methionine labeled crystalloid protein as a substrate. Optimum hydrolysis of [^{3}H]-leucine and [^{35}S]-methionine labeled crystalloid protein occurred at pH 5 and 37^{0}C. High levels of enzyme activity were observed in mature castor bean seed endosperm and these levels increased to a maximum two days after radicle emergence. The observed increase in enzyme activity appeared to be due to *de novo* synthesis, since it did not occur in endosperms that had been treated with cycloheximide. To identify hydrolysis products, reaction products in *in vitro* or soluble proteins in *in vivo* systems were fractionated by SDS-PAGE. Antibodies specific for crystalloid proteins, were used in western blotting experiments to study the hydrolysis of this storage protein. *In vivo* hydrolysis of crystalloid protein occurred in the following stages: 1) initial hydrolysis which occurred in the mature seed, yielded soluble polypeptides that appeared as homodimers in the 20 kD region of the gel, under reducing conditions; 2) by 6 hours after imbibition, profiles of another set of hydrolysis products, which appeared as heterodimers in the 20 and 30 kD regions of the gel, under reducing conditions, were obtained. This hydrolysis continued until 18

HAI; 3) after 18 HAI, the profile pattern changed and a new set of hydrolysis products appeared as homodimers in the 20 kD region of the gel, under reducing conditions. This continued until at least 96 HAI. However, in *in vitro* systems, a homodimer in the 20 kD region of the gel, under reducing conditions, was obtained. This profile pattern did not change for the 48 hrs of the experiment. Similar results were obtained when seeds were treated with 100 μM cyclohexamide which completely inhibited radicle emergence. We believe that an endopeptidase is involved in the initial mobilization of crystalloid protein reserves and further hydrolysis is due to both endopeptidases and exopeptidases. Evidence for this comes from initial hydrolysis products appearing in the 40 kD region of the gel while the native crystalloid protein banded in the 50 kD region of the gel. The absence of the other hydrolysis product of 10 kD on the gel, implies that it was rapidly hydrolyzed by endopeptidases and exopeptidases.

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ABBREVIATIONS

ABA	abscisic acid
BSA	bovin serum albumin
DAI	days after imbibition
GA	gibberellic acid
HAI	hours after imbibition
kD	kilodaltons
ME	β-mercaptoethanol
OD	optical density
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE	standard error

1. Introduction

1.1. The Castor bean plant

Castor bean plant (*Ricinus communis* L.), a member of the Euphorbiaceae is a widely adapted herbaceous shrub. It is a monoecious perennial that can grow to 13 m in the tropics and is believed to be indigenous to Africa (Schery, 1972, Simpson and Conner-Ogorzaly, 1986). Today, the plant is found wild and in cultivation throughout the world. The chief castor bean producing countries for commercial purposes are: India, Thailand, China, Mexico, the southern states of USA especially Oklahoma, Texas and Arizona, and Brazil which is the world's largest producer. In temperate areas, however, the plant is mainly grown as an ornamental annual. Both male and female reproductive organs are usually found on the same inflorescence, with androecium at the bottom and gynoecium at the top. This arrangement is probably an evolutionary adaptation to reduce self pollination. The castor bean chromosome number is $2n = 20$ although tetraploid cultivars have been bred in Africa. The castor bean plant fruit, before maturity, is globular with soft spines and is usually green or reddish-green. During fruit maturation, the outer covering shrinks into a dry, brown capsule that finally splits and sheds seeds, which are usually three in number.

1.2 The commercial significance of castor bean

The mature dry castor bean seed is oval in shape, fairly dark brown, mottled and has an average weight of 0.8g. The biochemical composition of the mature castor bean seed is approximately 6% water, 8% soluble carbohydrates, 16% fibre, 20% protein, and 48% lipids. The major seed storage lipids are triglycerides which yield glycerol and free fatty acids upon initial hydrolysis. Castor oil which is obtained from castor bean seeds, is a triglyceride ester of its fatty acids. Using gas chromatographic methods, the composition of fatty acids in castor oil, derived from the analysis of the methyl-esters of the fatty acids is as follows: 0.2% linolenic acid, 0.7-1.2% stearic acid, 0.9-1.2% palmitic acid, 1.3-1.4% di-hydroxy-stearic acid, 3.2-3.3% oleic acid, 3.4-3.7% linoleic acid and 89-89.4% ricinoleic acid (Hermann, 1974). Ricinoleic acid, is a fatty acid with 18 carbon atoms, one double bond in the ninth and tenth positions and one hydroxyl group adjacent to the twelfth carbon atom. This characteristic composition of the fatty acids is responsible for the physical and chemical properties that make castor oil different from all other seed oils. For example castor oil has a very high viscosity which varies very slightly at different temperatures, the highest density of all oils, high cold resistance and equally remarkable heat resistance, and finally, the ability to burn without residues. Because of these properties, it is used as a lubricant especially for rocket engines and airplanes (Simpson and Conner - Ogorzaly 1986).

Due to its polar hydroxyl groups, castor oil is compatible with numerous natural and synthetic resins, polymers and waxes, it is therefore used in the

manufacture of some soaps, synthetic rubber, imitation leather, plastics, and as an ingredient of nylon. It also has wetting and dispersing properties for pigments, filling agents and coloring matter, which make it useful in the manufucture of typewriter ink. Dehydrated castor oil, derived from heating the oil with acidic catalysts (eg. sulphuric acid), serves as a drying oil that is used in mixture with more expensive oils in the paint industry (Hermann 1974). Sulfonated castor oil is used as a wetting agent to promote color adherence during dyeing operations. Although castor oil is rarely used medicinally today, in the past it was used both in internal medicine and dermatology. At present, castor oil is more important in the cosmetics industry, where it is used as a softening agent in fingernail polishes, lipsticks, creams and lotions.

Castor oil is extracted by mechanically pressing the castor bean seed and allowing the oil to drip out into a container. The procedure is done with a screw press which generates heat that can reach temperatures between 65 and 72^{0}C (Simpson and Conner-Ogorzaly 1986). The castor bean seed also contains toxic compounds. When the oil is extracted, the toxins are often not removed from the seed. They remain in the residue. Consequently, the residue from castor oil extraction can contain enough toxic compounds to make it unsuitable for animal consumption, it is therefore used as a fertilizer.

1.2.1. The toxic properties of castor bean.

The castor bean seed contains the following compounds that are toxic to humans: ricine, which is a toxic alkaloid; ricin, a highly toxic protein; and a protein-polysaccharide complex (CB-A) which causes violent allergenic reactions in people that are sensitive to it. Ricin is a cytotoxic lectin. The several forms of ricin include ricin D, ricin E and the closely related *Ricinus communis* agglutinin. All forms of ricin are encoded by a small multigene family the expression of which is tissue specific and is developmentally regulated (Lord 1991).

Ricin is a heterodimeric protein that has a toxic polypeptide RTA or the A chain, linked by a single disulfide bond to a cell binding polypeptide, RTB or the B chain (Olsnes and Phil 1982). RTA, an N-glycosidase, catalytically inactivates susceptible ribosomes (e.g. mammalian or yeast ribosomes), by removing a specific adenine residue from a conserved stem-loop structure present in 26 to 28S ribosomal RNA (Endo and Tsurugi 1987). This makes the ribosomal RNA unable to bind elongation factors, consequently, the ribosome becomes irreversibly inactivated. A single RTA molecule can depurinate up to 2000 susceptible ribosomes per minute, which makes RTA a very powerful protein synthesis inhibitor. Ricin cytotoxicity is derived from RTA being covalently linked to RTB, which in turn is able to bind the holotoxin to the target cells. RTB, a galactose and N-acetylgalactosamine-specific lectin, binds to target cell surfaces by interaction with galactose-terminating oligosaccharides which are on many glycoproteins and glycolipids. The cell surface bound ricin is then internalized by receptor-mediated endocytosis via coated pits and endocytic vesicles (VanDeurs et al., 1985). RTA crosses the intracellular membrane,

following internalization, and enters the cytosol where it gains access to its ribosomal substrate. Plant ribosomes are less sensitive to RTA than their yeast or mammalian counterparts while bacteria ribosomes are insensitive to RTA action.

Ricin is a member of a larger group of plant toxins called ribosome-inactivating proteins (RIPs). At present a number of RIP genes including ricin are being cloned with the intention of introducing them into economically important plants to make them virus resistant (Pincus et al., 1990). This is possible since ricin and other RIPs have the potential to inhibit the transmission of plant viruses by selectively inhibiting ribosomal protein synthesis of viral-infected cells and in the process, preventing viral replication (Lord 1991). Ricin is presently being evaluated for treatment of HIV infections. Till et al., (1988) showed that soluble recombinant CD4 (a receptor for HIV), linked to RTA by a disulfide bond, selectively kills HIV-infected cells. Another therapeutic application of RTA is its use as a toxin moiety of immunotoxins (chemical conjugates in which a cell-reactive monoclonal antibody is linked to cytotoxin e.g. RTA, usually by a disulfide bond). While this application is used in the treatment of cancer where the selected target antigen is found on cancer cells but not on normal cells (Lord 1987), other chimaeric recombinant molecules have been produced by fusing DNA that encodes RTA to that that encodes other cell recognition proteins (Lord 1991).

1.3. Seed structure.

The mature castor bean seed is comprised of: 1) the embryonic axis, which lies in the central longitudinal position of the seed; 2) the endosperm, which completely surrounds the embryonic axis; and 3) the seed coat, which develops from the integuments that surround the ovule. The embryo has long, thin and flattened cotyledons which are in intimate contact with the endosperm tissue. These cotyledons do not contain large amounts of storage reserves. The majority of the storage reserves (>95%) are in the endosperm (Gifford et al., 1982). The endosperm surfaces that are in contact with the cotyledons have small invaginations which can be seen under dissecting microscopy, and although there is no experimental demonstration of this to date, it is possible that these surfaces contain transfer cells that are responsible for the translocation of mobilized reserves from the endosperm storage tissues to the embryo (Gifford, personal communication)

1.4. Food reserves: synthesis and storage.

During the course of seed development from the fertilized ovule to maturity, storage reserves are laid down. These storage reserves are stored in distinct subcellular organelles known as protein and lipid bodies. These reserves are laid down at one stage during seed development for future use during and following germination, where they will support protein synthesis and production of energy for early seedling growth until the seedling becomes fully autotrophic. The major food reserves of

castor bean seeds are lipids and proteins. These two reserves will be considered under separate sub headings.

1.4.1. The seed storage lipids.

Biological energy is most conveniently stored in the form of lipids because, unlike proteins and carbohydrates, lipids are relatively compact, anhydrous and have a very high calorific value (Murphy 1993). The major storage site for lipids in plants is in the seed. In oil seeds, including castor bean, storage lipids can account for more than half of the total weight of the mature seed, while in protein rich seeds eg. legumes, storage lipid content may be as low as 1-2% of the total seed weight. In most plants, storage lipids are in the form of triacylglycerols (Murphy 1990).

1.4.2. Synthesis of seed storage lipids.

Liposomes are synthesized from endoplasmic reticulum (Mayer and Marbach 1981). These organelles enclose the lipid reserves within the storage parenchyma cells. The subject of the origin and development of liposomes has been controversial but it is now believed that the newly formed lipids accumulate between the two layers of the ER membrane which leads to its swelling (Bewley and Black 1985). When the lipid-filled vesicle reaches a critical size, one of the following happens: 1) it may bud off the ER completely; 2) it may bud off with a little ER still attached to it; 3) it may retain multiple contacts with ER; or 4) it may completely come off as an independent "microsome" with the lipids deposited in the membrane. These lipid-filled vesicles

then form lipid bodies with storage fats accumulating between the phospholipid layers, and hence making the outer structure surrounding the mature lipid body a half-unit membrane.

1.4.3. The seed storage proteins.

According to classical seed protein definitions, seed storage proteins are divided into four classes by solubility (Osborne 1916): 1) **albumins** which are soluble in water and dilute buffers at neutral pH; 2) **globulins** which are insoluble in water but soluble in salt solutions; 3) **glutelins** which are soluble in acid and alkali solutions; and 4) **prolamins** which are alcohol soluble. Although this classification is not ideal, especially when modern reseachers use different extraction procedures particularly for quantitative purposes, a better one has yet to be devised. Globulins constitute a large class of 11S proteins that can be divided into the following subclasses: 1) *legumins* which are completely soluble in salt solutions and are restricted to the legumes; 2) *glutelins* which are restricted to cereals, and have slight solubility in salt solutions, slight solubility in acid and alkali solutions, 100% solubility in solutions containing urea or SDS; and 3) *crystalloids*. The crystalloids are found in castor bean and some conifers, and have minimum solubility in salt solutions, minimum solubility in acid and alkali solutions, about 90% solubility in urea solutions and 100% solubility in solutions containing SDS. The crystalloids are crystalline, proteinaceous inclusions that are embeded in the protein matrix in the protein bodies. The protein matrix is usually

made up of soluble 2S and 7S albumins. In the present study, the proteins that are dealt with, will be referred to according to the above descriptions.

1.4.4. Synthesis, processing, targeting and storage of seed storage proteins.

Rost (1972) used the types of proteins present to categorize angiosperm protein bodies into three structural types: 1) those consisting of amorphous protein without inclusions (peanut, soybean, and maize); 2) those with phytin globoids in protein matrix (Gossypium); and 3) those with both globoids and crystalloids (*Cannabis*, *Ricinus*, *Cucurbita*, *Yucca*). The globoid is a phytic acid storage inclusion. The crystalloid appears to be an ordered partly crystalline protein deposit (St. Angelo et al. 1968). Storage proteins are synthesized in the endosperm and deposited in the protein bodies during seed development (Ingversen 1975). The synthesis takes place in the cytoplasm on the endoplasmic reticulum (ER) to produce protein precursors. The protein precursors have a signal peptide that has a hydrophobic core which enables them to be sequestered into the lumen of the ER. The removal of the signal peptide by the action of an endopeptidase takes place in the ER. The protein precursors, due to their hydrophobic nature, form aggregates which are then secreted into the Golgi apparatus where post-translational modification e.g. glycosylation may occur. For example, the majority of the 7S lectins undergo glycosylation at this stage of development. The protein precursors are sorted into transfer vesicles at the trans-Golgi network. The vesicles are then targeted into the storage vacuoles, where further protein modification may take place. Many researchers agree that the translational

products of the 11S genes undergo post-translational modification in the protein bodies to produce mature 11S proteins (Bewley and Black 1985, Shannon and Chrispeels 1986, Muntz et al,. 1994). This modification, is a cleavage by a very specific endopeptidase, to form the acidic and basic polypeptide components of the 11S subunit. Cleavage occurs between an asparagine and a glycine residue and the latter becomes the N-terminal of the basic polypeptide. In some cases, further (secondary) modification occurs at the C-terminal of both the acidic and basic polypeptides. For example, Staswick et al,. (1984), after chemically determining the entire primary structure of the translational product of the glycinin gene of soybean using the mature protein, showed that four additional amino acids had been removed from the acidic polypeptide while five amino acids had been removed from the basic polypeptide. The sites of cleavage suggest the action of a protease which recognizes paired acidic amino acids. Examination of the primary structures of prolegumins and proglycinins, however, shows that some have tandemly linked basic residues where cleavage could occur, but others do not. Therefore the significance of secondary modification remains to be determined. Using ultrastructural studies of developing maize seeds, Khoo and Wolf (1970) also found that the endosperm protein bodies originate from vesicles produced by ER or formed at the enlarged ends of ER cisterna. Furthermore, Larkins and Hurkman (1978) showed that protein bodies of maize endosperm are a result of protein deposition associated with rough endoplasmic reticulum (RER) and the protein membranes are directly connected with RER.

1.4.5. Characterization of the major storage proteins of the castor bean seed

The major storage protein that make up 80% of the total protein reserve of castor bean are the 11S crystalloid proteins (Gifford et al. 1982, Tully and Beevers 1976). The castor bean crystalloids are a family of proteins and each holoprotein, whose mol. wt. range from 300 to 360 kD, is a homo-hexamer. Under denaturing conditions therefore, a family of subunits is obtained. Each of the six subunits has two polypeptide components, one with an acidic and the other with basic isoelectric point (Gifford and Bewley 1983). The 11S crystalloid subunits have molecular masses ranging from 49 to 53.5 kD with the molecular masses of the acidic and basic polypeptides that make up the subunit, between 29-34 kD and 20.5-23.5 kD respectively. The acidic and basic polypeptides are linked by a single disulfide bridge. The crystalloid subunits are not glycosylated (Gifford and Bewley 1983).

Salt-insoluble crystalloid proteins are also found in several species of the genera *Pinus* (Gifford 1988) and *Picea* (Gifford and Tolley 1989, Misra and Green 1990, Misra and Green 1991, Hakman et al. 1990). In a western blot analysis of 11S proteins from *Pinus pinaster* megagametophyte, Allona et al. (1992), using polyclonal antibodies raised against a 21 kD polypeptide, indicated that it was homologous with the 20-22 kD polypeptides from pea and soybean. In another study to show homology between the crystalloid proteins of conifer seeds and 11S globulins of angiosperms, Misra and Green (1994), using white spruce crystalloid protein antibodies, reported antigenic cross-reactivity with 11S proteins of several angiosperm species. The pattern of immunological cross-reactivity obtained suggests that the crystalloid

proteins of gymnosperms are more closely related to members of Euphorbiaceae, Brassicaceae, and Solanaceae. This is in doubt, however, since in the present study, using castor bean crystalloid protein polyclonal antibodies, such immunological relatedness was not found.

Studies of the 11S legumins from soybean (Tierney et al. 1987), field bean (Beachy et al. 1986) and pea (Goldberg et al. 1981, Hill and Breidenbach 1974) have shown that these proteins associate to form a hexameric structure, of about 360 kD mol. wt, similar to that of the crystalloid protein. The subunits in the legumin hexamer are not glycosylated and unlike the 11S crystalloids, they are heterogeneous. Other globulin-like proteins, eg. glutelins, have been shown to be related to the 11S proteins of angiosperms (Robert et al. 1985, Zho et al. 1983).

1.5. Dessication and processes that occur during seed maturation.

During seed development, storage reserve synthesis is terminated by seed desiccation which gives rise to a fully mature seed (Bewley and Marcus 1990). Kermode and Bewley (1986), showed that seed desiccation is involved in the suppression of storage reserve synthesis-related metabolism. Kermode et al. (1989) concluded that desiccation acts as a "switch" to suppress the transcription of genes for developmental proteins and allow the induction of other proteins, some of which will be needed in the process of germination. Evidence for this switch has been shown in many seed types: 1) castor bean (Kermode and Bewley 1985, Kermode et al. 1985,

Bewley et al. 1989); 2) soybean (Resenburg and Rinne 1988); 3) radish (Aspart et al. 1984); 4) bean (Bewley et al. 1989, Dasguta and Bewley 1982, Misra and Bewley 1985); and 5) wheat (Cornford et al. 1986). The changes in protein synthesis are produced by seed desiccation as a result of identifiable changes in mRNA populations. Specific changes in mRNA can be revealed by the use of complementary-DNA (cDNA) probes for the mRNAs in question; for example, phaseolin mRNA in French bean (Misra and Bewley 1985), and ricin D mRNA in castor bean (Kermode et al. 1989).

1.6 Quiescence and Dormancy

Seeds in which none of the germination processes are taking place, are said to be quiescent. Quiescent seeds are able to survive for long periods of time (hundreds and even thousands of years) in a resting state of low hydration level, and with very low metabolic activity (Bewley and Black 1985). For germination to occur, quiescent seeds are hydrated under suitable conditions such as the presence of oxygen and favorable temperatures.

Some seeds however, may have the right conditions for germination to occur but growth does not occur. These seeds are said to express dormancy. Seeds will show innate dormancy when they have an intrinsic block to the completion of germination as they are dispersed from their mother plants. Those seeds that develop the block due to environmental conditions are said to exhibit imposed dormancy. In

some cases, it is the seed coat that imposes dormancy by preventing water uptake, interfering with gas exchange and/or conferring mechanical restraint. This type of dormancy can be broken by fire (which raises the environmental temperature and in the process softens the seed coat), mechanical breaking of the seed coat, or by partial digestion of the seed coat by animals that ingest the seeds. In other seeds, dormancy is broken by certain "priming" treatments such as a light stimulus, or a wet period that is accompanied by cold or alternating temperatures. During such treatments, the block to germination is nullified and these conditions are no longer needed for the whole process to continue, but are only necessary for the initiation of the germination process. Once dormancy is broken and conditions for germination are favorable, then enzymes and mRNAs that have been lying in the seed in an inactive state are activated. Certain genes are also induced to encode specific enzymes and other cellular components that are needed for germination and early seedling growth (Bewley & Black 1985). In the study of the genetically imposed dormancy therefore, it is as important to determine the specific growth regulatory genes that are suppressed in these seeds, as it is to identify the growth maintenance processes that are affected by this dormancy.

1.7 Mobilization of storage reserves

1.7.1 Germination

In a non-dormant seed, germination begins with imbibition and ends with radicle emergence through the seed coat (Bewley and Black 1985, 1978). The immediate consequence of seed imbibition is an increase in ATP to levels that are high enough to initiate basic metabolic processes (Obendorf and Marcus 1974, Moreland et al., 1974). This implies that the process of germination is comprised of numerous events: 1) cellular hydration; 2) subcellular structural changes; 3) increased oxygen uptake and respiration; 4) synthesis of proteins and other cellular molecules; 5) cell division; and 6) cell elongation. For germination to occur, all these processes have to work in concert to transform a mature, resting seed, into one that is highly metabolically active, with the inevitable consequence of embryo growth (Gifford et al., 1983, Marcus and Rodaway 1982, Bewley and Black 1978, 1985).

Following germination, the most observable ultrastructural change is the disappearance of protein and lipid bodies from storage tissues. These ultrastructural changes are closely associated with protein and lipid reserve hydrolysis. The disappearance of storage organelles is also accompanied by appearence of proteins in the soluble fraction and high levels of amino acids in the embryonic axis (Gifford et al. 1983, 1986). Storage reserves are ultimately converted to small peptides, amino acids, sucrose and other macromolecular building blocks which are then transported to the embryonic axis. As will be seen later, the insoluble storage proteins are first mobilized by limited proteolysis to produce soluble intermediates that are further hydrolyzed to smaller peptides and amino acids. The amino acids can then be used for energy production and for the synthesis of nucleotides and proteins in the embryo. Upon

imbibition of the castor bean seed for example, the embryo immediately depletes its own reserves and before it becomes fully autotrophic, it relies on those reserves that are mobilized from storage tissues (Higgins 1984, Lammer and Gifford 1989).

1.7.2. Regulation of reserve mobilization during germination and early seedling growth

Studies in various species (Salmia 1981, Gifford et al. 1983, Lammer and Gifford 1989, Gifford and Tolley 1989, Groome et al., 1991), have shown that there is rapid hydrolysis of storage proteins following radicle emergence. Bewley and Marcus (1990) proposed that the numerous biochemical and physiological processes that are activated during germination and seedling growth are due to increased expression of different genes. The products of some of these genes are responsible for the higher metabolic processes that are achieved during this period, while other genes code for proteins that regulate these processes. In studying regulation of early seedling growth therefore, identification of genes that are expressed in the embryo during this time of development has been done, and in particular those genes whose expression are unique to seedling growth. DeVries et al. (1985) described a gene in 2-day-old pea seedlings that encodes a shoot-specific polypeptide. The absence of the mRNA (for this gene) from the plumule and the leaf, as well as the lack of an effect of illumination on its accumulation, eliminated its possibility of being related to the photosynthetic system. It was therefore suggested that it is involved in the elongation of the seedling stem. Expression of other mRNAs have also been identified in soybean (Datta et al. 1987),

pea (Lalonde and Bewley 1986), and canola (Harada et al. 1988) that are unique to seedling growth.

Reserve mobilization and early seedling growth are developmentally regulated both temporally and spatially. Evidence for this regulation is shown by the existence of preformed mRNAs, in mature seeds, synthesized during embryogenesis, which encode proteins that are synthesized following the onset of germination. Some of these preformed mRNAs decay rapidly during germination (Thompson and Lane 1980, Aspart et al. 1984). Other mRNAs populations that are newly transcribed following germination have been identified (Bewley et al. 1989, Lane and Thompson 1980, Peumans et al. 1982, Grzelczak et al. 1982, Litts et al. 1987, Lane et al. 1987). Possible methods to differentiate preformed mRNAs from the newly transcribed ones include the use of pulse-labeling experiments. One way in which this has been done was to allow newly synthesized mRNAs to incorporate thiouridine, thereby separating newly synthesized from preformed mRNAs (Stetler and Thorner 1984, Cramer et al. 1985). Using specific probes, whose selection was based on their presence in both mature seeds and germinating seeds, levels of specific mRNAs in these two populations were then quantified. In another approach, Masumori et al. (1992) using RNA blot hybridization techniques, identified stored mRNAs in dry seed of *Pinus thunbergii* and while one of the mRNAs disappeared rapidly during germination, others were still detected until the end of germination, which suggests regulation of gene expression. More evidence for this has been shown by other studies (Harada et al. 1988a, Misra and Bewley 1985, Harada et al. 1988b, Dietrich et al. 1989, Datta et

al. 1987, Lalonde and Bewley 1986). The changes, which can be shown by protein electrophoretic analysis, have been investigated at different times of development, in different seed types during germination and seedling establishment (Gifford et al. 1991, Gifford et al. 1984, Rosenberg and Rine 1989, Marcus and Rodway 1982, Dure et al. 1981). Some of these proteins are proteolytic enzymes that mobilize proteins and lipids during germination and early seedling growth.

When activities of these enzymes are in measurable magnitudes to allow for biochemical analysis, they can be used to study regulatory processes. For example, the accumulation of amylases and proteases that brings about the rapid hydrolysis of the abundant storage components in the cereal endosperm is controlled by gibberellic acid (GA) and abscisic acid (ABA) (Bewley and Marcus 1990). Following germination, the cells of the aleurone layer of wheat and barley synthesize several hydrolytic enzymes, the most abundant being α-amylase (Baulcombe and Buffard 1983). Activation of aleurone layer cells to produce α-amylase is due to the synthesis of GA in the embryo and its subsequent release into the aleurone layer. Zwar and Hooley (1986) and Deikman and Jones (1986), showed that GA and ABA influence the transcription rates of α-amylase genes in aleurone protoplasts of wild oat. Hammerton and Ho (1986) identified a carboxypeptidase in barley aleurone layer, whose secretion but not accumulation depends on GA.

Since the products of hydrolysis of storage reserves are used for seedling growth, there should be a direct coordination between storage reserve hydrolysis, the enzymes responsible for this hydrolysis, and the growth of the seedling. Consequently,

the regulatory role of embryonic axis in reserve hydrolysis has been investigated further in many other species. It has been shown that removal of the embryonic axis before imbibition impairs the normal hydrolysis of storage reserves in cucumber (Davies and Chapman 1979, Slack et al. 1977), bean (Kern and Chrispeels 1978, Minamikawa 1978, Morohashi 1982) and pea (Chin et al. 1972). Gifford et al. (1984) showed that the embryonic axis is required for both rapid protein breakdown and the attainment of high levels of activity of proteolytic enzymes in the castor bean endosperm. However, in castor bean it was shown that the embryonic axis is only involved in the initiation of these processes but not in their maintenance. Ching (1970) and Nyman (1971) have also shown that embryo attachment is necessary for the normal reserve mobilization in gymnosperm megagametophytes. Since gibberellic acid has been found to play a role in proteolytic enzyme induction, it is possible that the embryo axis controls the mobilization of reserves by producing plant hormones which are then transported to the storage tissues. The consequent result is the induction of the proteolytic enzymes in the storage tissues that hydrolyze storage reserves. Mullen and Gifford (1995) reported that the embryo axis affects the levels of catalase enzyme activity and the steady-state protein in cell-free extracts of endosperms during germination and seedling growth in castor bean seeds, although Huang and Beevers (1974) did not find any effect of embryo axis on reserve mobilization.

1.8 The role of endopeptidases in mobilization of protein storage reserves

Many studies, including the present study, suggest that endopeptidase activity is a necessary prerequisite for the rapid mobilization of the reserve proteins which accompanies germination. A major cysteine endopeptidase, designated SH-EP, was found to be expressed in cotyledons of germinating *Vigna mungo* seeds (Mitsuhashi et al., 1986, Akasofu et al., 1989, Mitsuhashi and Minamikawa, 1989). Another endopeptidase from pods of *Phaseolus vulgaris* fruits, which was designated EP-C1, was found to be immunologically identical to SH-EP (Tanaka et al. 1993). The activity of this endopeptidase (EP-C1), but not of exopeptidases, was found to increase continually throughout the development and subsequent senescence of the pods of the *Phaseolus vulgaris* plants (Endo et al.1987, Tanaka et al 1989). RNA blot hybridization with EP-C1 cDNA as a probe showed that EP-C1 mRNA occurred in the pods of fruits at later maturing stages as well as in the cotyledons of germinating seeds. The endopeptidase activity was therefore thought to be involved in the protein mobilization in pods during fruit maturation as well as in germinating seeds.

Chrispeels and Boulter (1975) found that a sulfhydryl-type endopeptidase activity was responsible for initial hydrolysis of reserve proteins in mung beans. Boylan and Sussex (1987) purified an endopeptidase that is involved in storage-protein degradation in cotyledons of *Phaseolus vulgaris* L. Mainguy et al (1972) had identified a serine-type endopeptidase in peanut seeds while in germinating seeds of vetch, an asparagine-endopeptidase was found (Shutov et al. 1982). A similar enzyme was found in germinating kidney beans (Csoma and Polgar 1984). Specificity studies suggest that, like its counterpart in developing seeds, the enzyme in the germinating

seeds will only hydrolyze peptide bonds formed by the carboxyl group of asparagine (Shutov et al. 1982). Since asparagine-endopeptidases apparently act on different asparagine residues of the same proteins in both developing and germinating seeds, these enzymes are highly regulated throughout seed development and germination. These enzymes process protein precursors into mature proteins during seed development, and as well, hydrolyze the mature storage proteins for the purpose of seedling growth. Yu and Greenwood (1994) found that there was a cysteine endopeptidase that is involved in globulin hydrolyzation in germinated *Vicia faba* L. seeds. They found that this protease was involved in the initial modification of the storage proteins *in vivo*. A specific endopeptidase activity is therefore a necessary requirement for the initiation of mobilization of storage reserves and thus providing intermediates for the subsequent rapid and more extensive reserve hydrolysis by less specific endopeptidases and exopeptidases.

1.9 The present study

In castor bean seeds, the mobilization of lipid reserves has been extensively studied, however, far less is known about storage protein mobilization. Although the studies on enzymes that are involved in storage protein hydrolysis have been published, these studies have used artificial chromogenic substrates for the enzymes and therefore the nature of hydrolysis of the native storage proteins by these enzymes is unclear (Gifford et al., 1983). This study was therefore undertaken to identify the

enzymes involved in native protein reserve mobilization in castor bean during germination and seedling establishment. To do this, it was necessary to: 1) design an assay system for the enzyme(s) that hydrolyze native storage proteins, using the actual storage protein (crystalloid protein) as a substrate for the enzyme(s); 2) study the kinetics of this enzyme(s); and 3) find out if the enzyme activity and the subsequent reserve hydrolysis are due to enzyme activation of existing hydrolases, the disappearance of inhibitors, or enzymes that are synthesized *de novo*.

Furthermore, a study of the changes (quantitative & qualitative) in protein reserves during seedling establishment was undertaken, and using western blot analysis, the steps involved in hydrolysis of storage proteins were shown. Both *in vivo* and *in vitro* systems were studied. The effect of cycloheximide (a protein synthesis inhibitor) on storage protein hydrolysis was also studied.

Finally, immunological relatedness of 11S proteins from seeds of different species (*Pisum sativum*, *Pinus taeda* L., (*Pseudotsuga menziesii* [Mirb] Franco), *Picea glauca* L., *Phaseolus valgaris*, *Brassica napus*, and *Ricinus communis* L.) was established in a western blot analysis using crystalloid protein polyclonal antibodies from castor bean. Using castor bean crystalloid antibodies, elicited to the reduced and non-reduced crystalloid proteins, an attempt was made to determine the structural conformation of crystalloid holoprotein using immunoblot analysis.

II MATERIALS AND METHODS

2.1 Plant material and Germination

Castor bean seeds (*Ricinus communis* L. cv. Hale) were obtained commercially from Bothwell Enterprises (Plainview, TX, USA). Seed coats were removed from mature seeds and the seeds were then allowed to imbibe on moist kimpak (Seedboro Equipment, Chicago, IL, USA) lined petri dishes, in the dark at 30°C. Endosperms were harvested at the required stages and both soluble and insoluble proteins were extracted for analysis.

2.1 Chemicals

Electrophoresis and research grade chemicals were purchased from Fisher Scientific (Edmonton, AB, Canada), Sigma Chemical Co. (St. Louis, MO, USA) and Amersham (Oakville, ON, Canada). [^{3}H]-labeled leucine, [^{35}S]-labeled methionine, [^{14}C]-labeled methylated molecular weight standards, and aqueous counting scintillant (ASC-II) were obtained from Amersham. Non-radioactive low molecular weight standards were obtained from Bio-Rad Laboratories (Mississauga, ON, Canada). DEAE-sepharose and CM-cellulose resins were purchased from Sigma Chemical Co. In this study, all water used was milli-Q quality (Pharmacia LKB).

2.3 Equipment

The J2-21/E centrifuge, Microfuge E, LS 6000TA Scintillation counter, scintillation vials and Du-65 spectrophotometer were commercially obtained from Beckman (Richmond, BC, Canada). The mini-protean II dual slab cell and the gel dryer were obtained from Bio-Rad Laboratories. The fraction collector was purchased from Pharmacia.

2.4 Protein Extraction

Protein extraction and subsequent separation of soluble and insoluble fractions were done according to Gifford et al. (1982), with modifications. All procedures were carried out at 4°C. Castor bean endosperms were ground in a mortar in 0.05 M sodium-phosphate buffer (pH 7.5) containing 0.10 mM leupeptin (a protease inhibitor), in a ratio of 1 seed to 1 ml extraction buffer. The homogenate was centrifuged at 18000 rpm (25300 g) for 20 minutes. The supernatant, containing soluble proteins, was removed and the pellet was reextracted three times, centrifuging at 18000 rpm (25300 g) for 5 minutes each time. The insoluble proteins in the pellet were solubilized by boiling the pellet for 5 minutes in Laemmli buffer, 65 mM Tris-HCl (pH 6.8) buffer containing 2% (w/v) SDS and 10% (w/v) glycerol (Laemmli 1970). A ratio of 1 seed to 1 ml extraction buffer was used. Whenever it was required, 5% (v/v) ME was added after boiling to reduce disulfide bridges. After this treatment, the

slurry was centrifuged at 18000 rpm (25300 g) for 20 minutes, and the supernatant containing the insoluble proteins was removed. Quantitative protein determination was done by the method of Lowly et al., (1951), using BSA as standard.

2.5 Isolation and fractionation of protein bodies

Protein bodies were isolated from mature castor bean endosperms. The non-aqueous method of Yatsu and Jacks (1968) was used with modifications. The procedure was carried out at room temperature (22^0C). Castor bean seeds were deshelled and embryos and cotyledons were removed. The endosperms were chopped in fine slices with a razor blade and gently ground in a mortar and pestle, using a ratio of one seed endosperm to one ml of glycerol. The slurry was strained through a cheese cloth into a centrifuge tube and centrifuged at 3000 rpm for 5 minutes. The pellet was discarded and the supernatant was re-centrifuged at 18000 rpm (25300 g) for 20 minutes. The supernatant and the encrusting lipid layer were removed. The pellet was re-extracted in glycerol three times, recentrifuged, and all supernatants removed, leaving a fat-free pellet. To remove glycerol from the pellet, the pellet was resuspended in 100% ethanol and centrifuged in a bench-top clinical centrifuge at 3500 rpm for 5 minutes and the supernatant was removed. This was repeated 3 times and the pellet which was now glycerol-free, was dried gently in an air stream to remove traces of ethanol. The subsequent fractionation was done according to Tully and

Beevers (1976). All the fractionation procedures were carried out at 4°C. The protein body pellet was resuspended in 5 mM Tris-HCl (pH 8.5), cooled on ice for 5 minutes, then centrifuged at 18000 rpm (25300 g) for 20 minutes. The supernatant which contained the buffer-soluble "matrix" proteins was removed and the pellet which contained buffer insoluble crystalloid proteins was washed 3 times in the 5 mM Tris-HCl. The crystalloid protein pellet was solubilized according to further use.

2.6 Ion Exchange Chromatography

The crystalloid protein complex was purified on a CM-Sepharose (27 by 1.5 cm) column according to Gifford et al (1983), with modifications. All procedures were carried out at room temperature (22^0C). Crystalloid protein (approximately 90 mg) was dissolved in 7 ml of acetate-urea buffer (5 mM Na-acetate [pH 5] containing 6M urea). This solution was loaded on the CM-Sepharose column that had been equilibrated with the acetate-urea buffer. The column was washed with 50 ml acetate-urea buffer and the bound proteins were eluted with a 200 ml linear NaCl gradient (0-0.3 M) in acetate-urea buffer. Three ml fractions were collected, at a rate of 25 ml per hr. The protein elution profile was estimated by following the absorbance at 280 nm. Fractions containing pure crystalloid protein were pooled and dialyzed overnight against water. The precipitated crystalloid protein was dried gently in air and the resulting powder was stored at -20^0C.

Soluble matrix proteins were purified on a DEAE-celullose (27 by 1.5 cm) column according to Tully and Beevers (1976) with modifications. All procedures were carried out at 4^0C. Whole mature seeds were extracted in 5 mM Tris-HCl (pH 8.5) at a ratio of 1 ml per seed. The seeds were ground in buffer and the homogenate was centrifuged at 18000 rpm (25300 g) for 20 minutes at 4^0C. The supernatant was collected and kept on ice until needed. Seven ml of this solution containing approximately 90 mg protein were loaded on the column that had been equilibrated in the Tris-HCl buffer. The column was washed with 60 ml of the buffer and the bound proteins were eluted with a 300 ml linear NaCl gradient (0-0.3 M) in the Tris-HCl buffer. Three ml fractions were collected at 4^0C, at a rate of 25 ml/hr. The protein elution profile was followed by monitoring the absorbance at 280 nm. The profile showed 3 peaks, and fractions from each peak were boiled for 5 minutes in equal volume of Laemmli buffer (Laemmli 1970) and the protein patterns determined by electrophoresis.

2.7 Polyacrylamide Gel Electrophoresis

Single-Dimension SDS-PAGE was carried out in 0.75 mm 12% acylamide slab gels on a Bio-Rad Laboratories mini gel system, according to Laemmli (1970). The molecular weights of proteins were determined by the method of Weber and Osborne (1969). Molecular weight standards included lysozyme, 14.4 kD; soybean trypsin,

21.5 kD; bovine carbonic anhydrase, 31 kD; hen egg white ovalbumin, 45 kD; bovine serum albumin, 66.2 kD; rabbit muscle phosphorylase b, 97.4 kD.

2.8 Staining and Fluorography

Following electrophoresis non-radiolabeled protein gels were stained with coomassie brilliant blue R for 30 minutes as in Burk et al. (1983), and then destained with two 60 minute washes of a solution containing 7% acetic acid and 8% methanol in water. The stained gels were dried on filter paper at 80^0 C for 2 hrs on a Bio-Rad gel dryer. For autoradiography, gels containing radiolabeled proteins were soaked in a solution containing aqueous 30% methanol and 7% acetic acid. The gels were then shaken for 2 hrs in 200 ml En^3Hance and then shaken in water for 10 minutes. All gels were dried under vacuum for 2 hrs at 60^0 C on a gel dryer. Gels containing radiolabeled proteins were exposed to X-ray film (X-omat AR-5) at -70^0 C (Gifford and Bewley 1984). The [^{14}C]-methylated molecular weight standards used were: lysozyme, 14.3 kD; carbonic anhydrase, 30.0 kD; ovalbumin, 46.0 kD; bovine serum albumin, 69.0 kD; phosphorylase b, 92.5 kD myosin, 200.0 kD.

2.9 In-vivo protein labeling

Castor bean plants were grown in the greenhouse at approximately 24^0C day, 18^0C night temperatures, a 16 hr photoperiod and approximate relative humidity of

80%. Seed staging was done according to the timetable established by Greenwood and Bewley (1981). Under our growth conditions, maximum storage protein accumulation in the endosperm occurred 30 days after flowering (DAF). Seeds were taken at this stage, deshelled, and the cotyledons were removed. Isolated endosperm halves were then placed in a Petri dish with the surfaces formerly in contact with the cotyledons facing up. Tritiated leucine (10 μCi in 50 μl per half seed endosperm) was applied to these surfaces, and protein labeling was allowed to occur for 6 hrs in the light at 30^{0}C. The insoluble crystalloid proteins were extracted as described in section 2.4 and the resulting powder was stored at 4^{0}C.

2.10 Enzyme assay

The method of Boylan and Sussex (1987) was used with modifications. The assay was based on the fact that, while the crystalloid protein is insoluble, the products of its hydrolysis are soluble in aqueous solutions (Gifford et al. 1986). The enzyme extract was prepared in the same manner as for the buffer soluble proteins described in section 2.4, except that a 25 mM citrate-phosphate buffer containing 5 mM ME at pH 7.2 (without leupeptin) was used. The enzyme activity was assayed at 37^{0}C in a 3 ml reaction mixture containing 0.01 g of substrate ([^{3}H]-leucine labeled crystalloid protein, approximately 200000 dpm), 1 ml enzyme extract, 2 ml of 0.1 M citrate-phosphate buffer (pH 5) that contains 5 mM ME and 0.1 M chloramphenical. The mixture was incubated for the required time with constant shaking on a nutator. The

reaction was stopped by centrifugation at maximum speed in a bench-top clinical centrifuge and 1 ml of the supernatant was counted on the scintillation counter. Specific activity was expressed as dpm/hr/mg protein.

2.11 Antibody production

Polyclonal antibodies to the crystalloid protein were produced by injecting 200 µg of the purified protein in 0.75 ml of 8 M urea with or without 0.2% ME, and 0.75 ml of Freunds complete adjuvant subcutaneously and submuscularly at multiple sites of female rabbits that were a cross between French lops and Flemish giants. Two separate booster injections were administered every 14 days. These injections were similar to the initial injections except that the protein was in 0.75 ml 8 M urea with or without 0.2% ME and 0.75 ml of Freunds incomplete adjuvant. Rabbits were bled 8 days after every booster injection to test for titer. Blood was drawn, incubated at 37^0C for two hrs and then at 4^0C overnight. The blood was centrifuged at maximum speed in a bench-top clinical and the serum was aliquoted and stored at -70^0C.

2.12 Western blot analysis of proteins

After SDS-PAGE, gels were equilibrated for 17 minutes in transfer buffer (25 mM Tris, 192 mM glycine and 20% v/v methanol), containing 0.015% SDS. The transfer of proteins to nitrocellulose (Bio-Rad) was carried out overnight at 4^0C at a

constant voltage of 30 V in a Mini Trans-Blot Cell (Bio-Rad) according to the manufacturer's recommendations. The efficiency of protein transfer to the nitrocellulose membrane was measured by Ponceau S staining as described in Sambrook et al. (1989). To visualize the transferred proteins, the membranes were stained for 10 minutes in a solution that contained 2% (w/v) Ponceau S, 30% (w/v) trichloroacetic acid and 30% (w/v) sulfosalicylic acid in water, and then destained in 3 washes of water. The subsequent immunoblotting was done using the method of Kunce and Trelease (1986). Blots were blocked for 1 hr at room temperature in "blotto", which is 3% (w/v) non-fat (Carnation) dry milk, 0.15 M NaCl and 20 mM Tris-HCl (pH 7.8). The blots were incubated with primary antibody for 2 hrs at room temperature at a ratio of 1:5000 and then washed 3 times in the "blotto" solution described above (20 minutes each wash). The secondary alkaline phosphatase-goat-anti-rabbit antibody (Sigma Chemical Co.) was added and incubated for 2 hrs at room temperature in the above "blotto" solution, at the a ratio of 1:1000. After three 20-minute washes, cross-reactive proteins were visualized using alkaline phosphatase activity according to the manufacturer's instructions (Bio-Rad).

2.13 Immunoprecipitation

After incubation of [^{35}S]-methionine labeled crystalloid protein with the enzyme extract, the storage protein hydrolysis products were immunoprecipitated Using the combined methods of Harlow and Lane (1988) and Sambrook et al. (1989),

10 µl of the reaction products (300000 dpm) were brought up to 1 ml in a solubilization buffer that contains 1% Triton X-100 (v/v), 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 20 mM methionine, 3 mM EDTA, and 2µg/ml apopritinin. After standing on ice at 4°C for 15 minutes this mixture was then centrifuged for 15 minutes at 12000 rpm at 4^0C, 1 M solid NaCl was added to the supernatant. A 20 µl aliquot of 10% *Staphylococcus aureus* Cowan I cells (Sigma Chemical Co.) which had been washed in the above buffer, was added to the above mixture. After incubation for 1 h at room temperature, the mixture was centrifuged for 15 minutes at 12000 rpm at 4°C. The supernatant was removed and to it was added 20 µl of crystalloid protein antibody and mixture incubated on a nutator for 1 h at room temperature and then overnight at 4^0C. Immune complexes were collected by addition of 20 µl of *Staphylococcus aureus* Cowan I cells. After a 1 h incubation at room temperature with constant mixing on a nutator, cells were collected by centrifugation at 12000 rpm for 15 minutes at 4^0C. The cells were washed 5 times in a modified solubilization buffer which contained 0.1% Triton X-100. The cells were further washed 3 times in a modified solubilization buffer that lacked Triton X-100. Immune complexes were dissociated by boiling in 30 µl of Laemmli buffer at 95^0C for 5 minutes (Laemmli, 1970) and then centrifuged at 12000 rpm for 15 minutes at room temperature. An aliquot of the resulting supernatant was analyzed by SDS-PAGE and the immune complexes were visualized using fluorography. The remainder of the supernatant was stored at -70^0C until needed.

2.14 In vivo analysis of storage protein hydrolysis

Soluble proteins were extracted, as described in section 2.4, at different stages of seed imbibition [0, 6, 12, 18, 24, 48, 72 and 96 hours after imbibition (HAI)] and then boiled in Laemmli buffer in a ratio of 1:1 (v/v) for 5 minutes at 95°C. After SDS-PAGE, the proteins were transferred on to nitrocellulose and the storage protein hydrolysis products were subsequently analyzed by western blot analysis using crystalloid protein antibody. In some cases, 5% ME was added to the Laemmli buffer so that the reduced form of the storage protein hydrolysis products could be analyzed. In another experiment to study the effect of cycloheximide on storage protein hydrolysis, seeds were imbibed as described in section 2.1 except that 100 mM cycloheximide in water. The resulting products of hydrolysis were then analyzed by western blot analysis, following SDS-PAGE.

2.15 In vitro analysis of storage protein hydrolysis

Non-radiolabeled crystalloid protein complex was used in a reaction mixture as described in section 2.10, except that the enzyme extract used was obtained from mature seeds. The resulting soluble reaction products were then analyzed for storage protein hydrolysis products using western blotting, following SDS-PAGE.

111 Results

3.1 Germination

Figure 1 shows the relationship between the radicle length of the castor bean seed and DAI, when the seed was imbibed in pure water, 10 μM cycloheximide, or 100 μM cyloheximide at 30°C. Radicle length was measured daily during early seedling growth. When the seed was imbibed in pure water, radicle emergence, which determines the end of germination, occurred between 12 and 24 hrs after imbibition. Radicle length increased steadily until 5 DAI, when visible decay of the endosperm, which accompanied senescence, occurred. When the seed is imbibed in 10 μM cycloheximide, radicle emergence occurred approximately 1 DAI but the radicle length did not increase any further. However, there was no radicle emergence when the seeds were imbibed in 100 μM cyloheximide.

3.2 The kinetics of the enzymes that hydrolyze [^{3}H]-labeled crystalloid proteins

3.2.1 Time course of enzyme activity

Figure 2 shows the time course of the activity of enzymes hydrolyzing [^{3}H]-leucine labeled crystalloid protein. Enzyme activity was measured in dpm per hour per mg protein. Activity increased steadily from 0 dpm to 30000 dpm over 30 hrs, then it leveled off at 48 hrs. The results show that the rate of [^{3}H]-leucine labeled crystalloid protein hydrolysis was highest between 10 and 15 hrs.

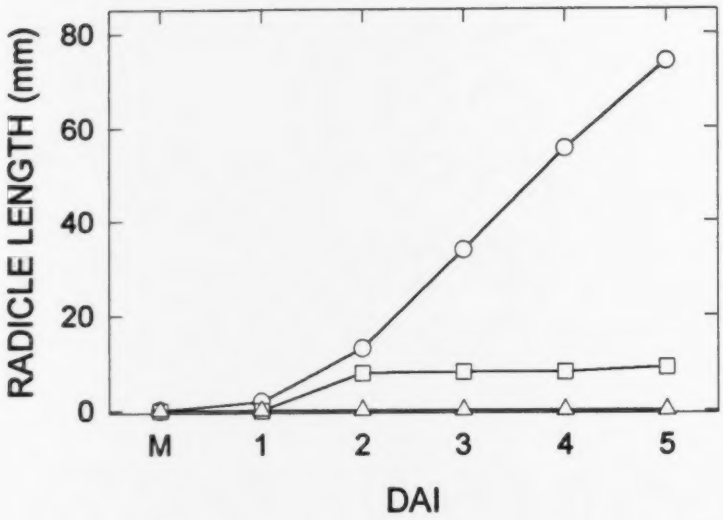


Figure 1

Changes in radicle length following seed imbibition at 30^{0}C. (o) seed imbibition in water, (□) seed imbibition in 10 μM cycloheximide, (Δ) seed imbibition in 100 μM cycloheximide. Each data point is a mean of 20 determinations ± S.E. Values on the X-axis are (M) mature seed, (1-5) DAI.

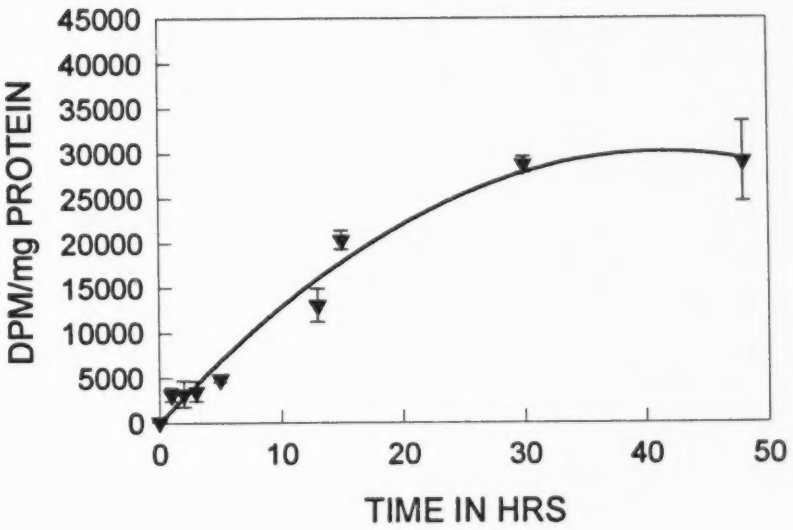


Figure 2

Time course of the activity of enzymes hydrolyzing [^{3}H]-leucine labeled crystalloid protein. The enzyme extract was obtained from seeds harvested 3 DAI. Each data point is a mean of 3 determinations ± S.E.

3.2.2 Effect of pH on enzyme activity

Effect of pH on the activity of enzymes hydrolyzing the crystalloid protein complex is shown in Figure 3. The enzyme extract was obtained from seeds harvested 3 DAI. The figure shows that there was measurable enzyme activity between pH 2.5 and pH 8.8. Optimal enzyme specific activity of 35000 dpm/hr/mg protein was obtained at pH 5.

3.2.3. Effect of protein concentration on enzyme activity

The relationship between activity and protein concentration was investigated. As shown in Figure 4, activity increased with increase in protein concentration. There was no enzyme activity when the reaction mixture included an enzyme extract that had been boiled.

3.2.4 Changes in enzyme activity following seed imbibition

Figure 5 shows that cell free extracts of endosperms taken from mature seeds, contained high levels of the enzyme activity. This activity increased to a maximum two days after radical emergence. The native crystalloid protein decreased quantitatively with increase in enzyme activity, also shown in this figure.

3.2.5. The effect of cycloheximide on the activity of enzymes that hydrolyze storage proteins

Figure 6 shows that in the presence of cycloheximide (100 μM), there was no increase in hydrolytic enzyme activity following seed imbibition. This decrease contrasted with the increase in enzyme activity in the absence of cycloheximide, also shown in this figure. The low enzyme activity seen in Figure 6, when compared to Figure 5, was due to a lower specific radioactivity of the crystalloid protein substrate that was used in this experiment.

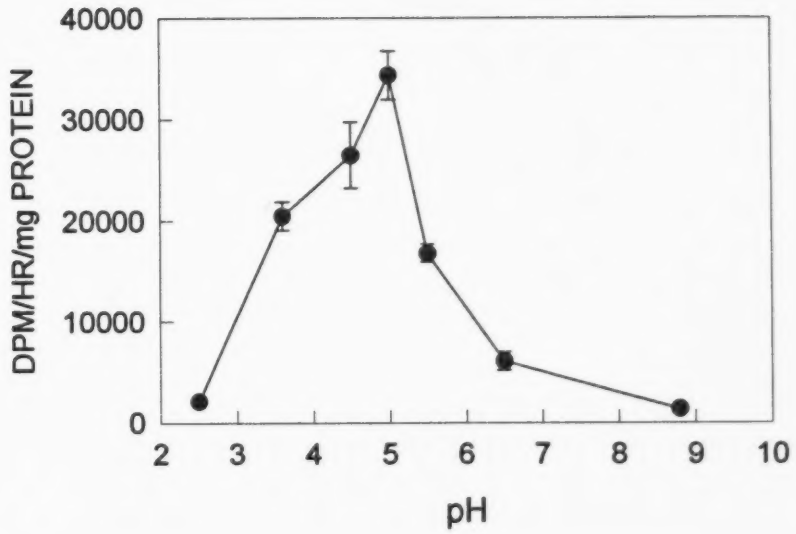


Figure 3

Effect of pH on hydrolytic enzyme activity. The enzyme extract was obtained from seeds harvested 3 (DAI). Each data point is a mean of 3 determinations ± S.E.

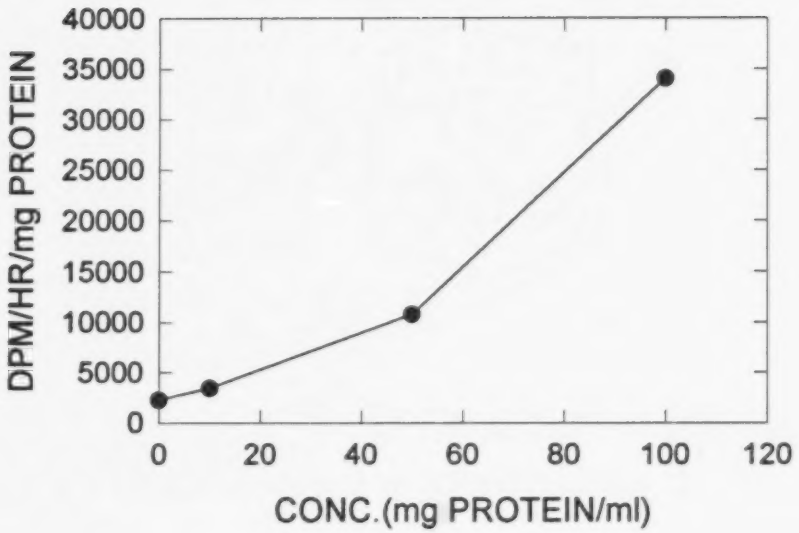


Figure 4

Effect of protein concentration on enzyme activity. The enzyme extract was obtained from seeds harvested 3 DAI. Each data point is a mean of 3 determinations ± S.E.

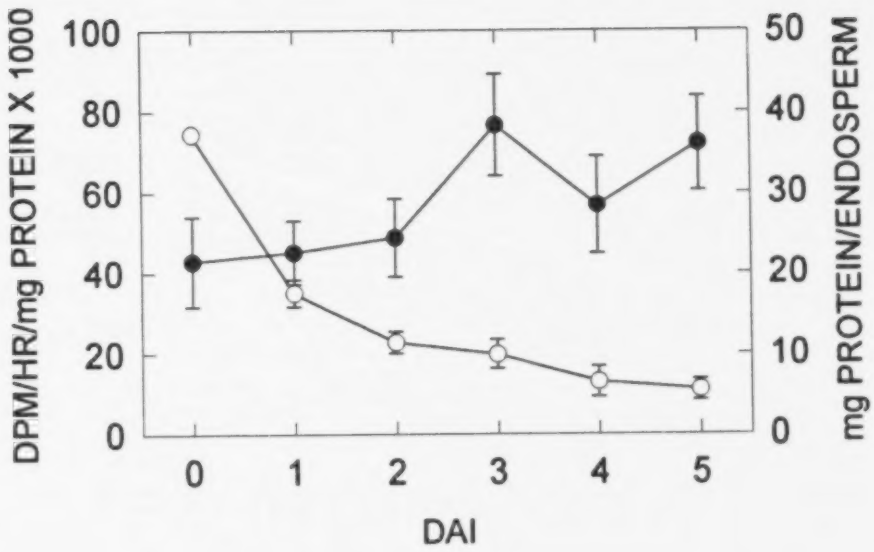


Figure 5

Changes in enzyme activity following seed imbibition (•) and the corresponding quantitative change of endospermic insoluble storage proteins (o). Each data point is a mean of 3 determinations ± S.E.

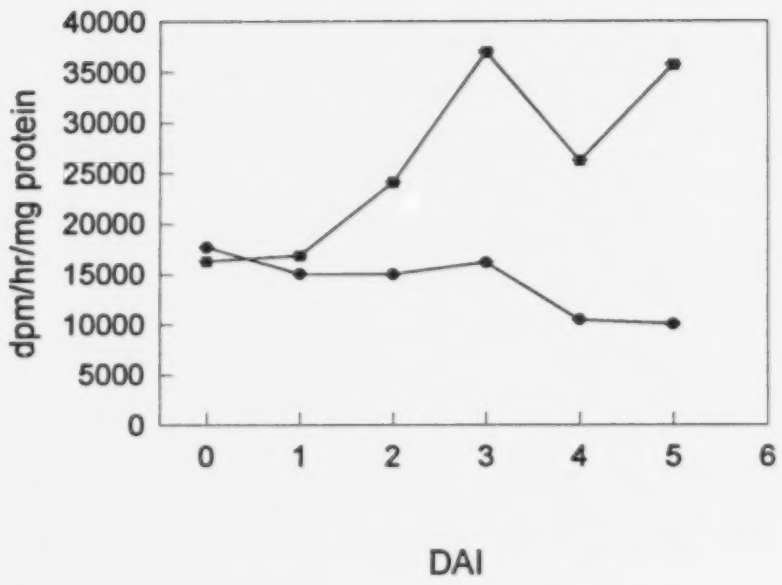


Figure 6

The effect of cycloheximide on the activity of enzymes that hydrolyze storage proteins. The Figure shows changes in enzyme activity, in the presence of cycloheximide (•) and in the absence of cycloheximide (▮). Each data point is a mean of 3 determinations ± S.E.

3.3 Purification of the crystalloid protein from castor bean endosperm

3.3.1 Elution profile of protein body extract on CM-sepharose column, and SDS-PAGE analysis of eluted proteins.

Figure 7 shows that chromatography on a column of CM-sepharose separated the protein body preparation into 3 peaks. Fractions representing each peak were pooled and dialyzed against 5 mM sodium acetate (pH 5). The precipitated proteins were dissolved in Laemmli buffer (Laemmli 1970) and analyzed by SDS-PAGE. The prominent peak that came off the column after the application of the salt gradient (fractions 40-44) showed the characteristic electrophoretic pattern of the crystalloid protein complex as described by Gifford and Bewley (1983). This pattern is shown in Figure 8. Under non-reducing conditions, (Figure 8 A), minor bands were seen in the 100 kD region of the gel with a major band in the 50 kD region of the gel. Under reducing conditions, (Figure 8 B), two groups of bands one in 30 kD region of the gel and the other in 20 kD region of the gel were obtained.

3.4 Production of crystalloid protein antibodies

Polyclonal antibodies were raised against the crystalloid proteins that had been either solubilized under reducing (+ME) or non-reducing conditions. To examine the specificity of these antibodies against native crystalloid proteins, crystalloid proteins were separated by SDS-PAGE, transferred to nitrocellulose and then probed with the antibodies. Figure 9 shows a western blot analysis of the crystalloid proteins. In (A), antibodies raised to the non-reduced protein were used while in (B) antibodies raised to the reduced protein were used. In both figures, lanes 1 and 2 show gel protein profiles following SDS-PAGE under non-reducing and reducing conditions

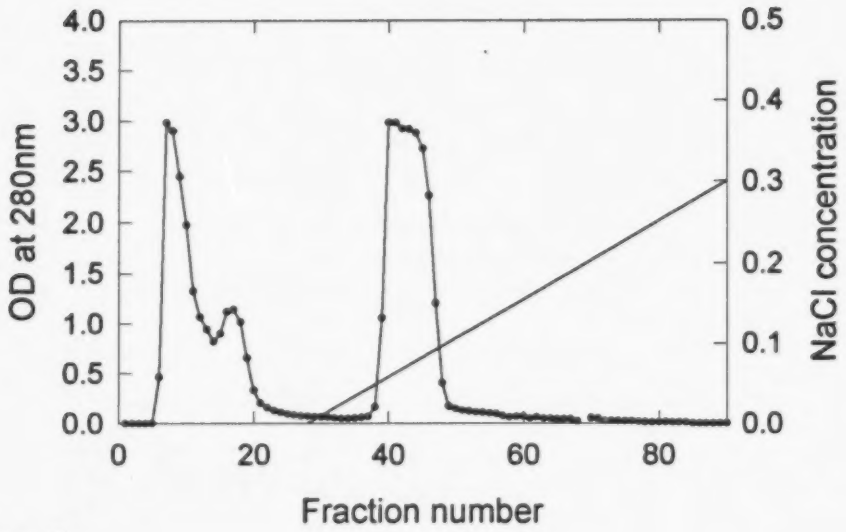


Figure 7

Elution profile of crystalloid proteins (•) separated by CM-cellulose ion-exchange chromatography viewed by absorbance at 280 nm. Also shown is the NaCl gradient (-) used to elute the bound proteins. Three ml fractions were collected.

Figure 8

SDS-PAGE profiles of crystalloid protein, run under (A) non-reducing conditions and (B) reducing conditions (+ME). In each case, 2 μg of crystalloid protein was applied to the gel. Numerical values are mol wt in kD.

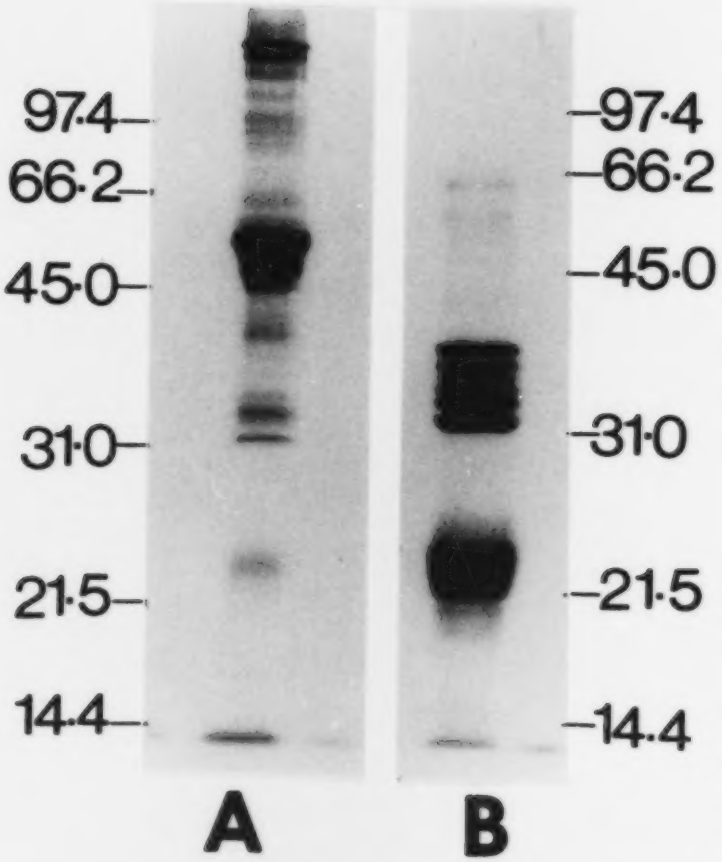
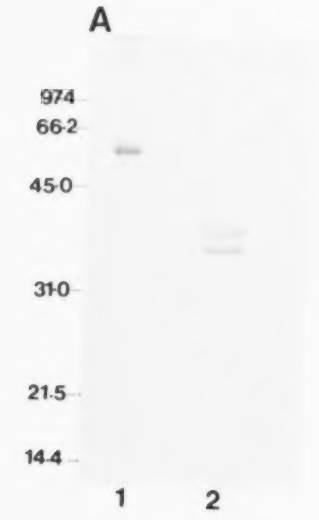
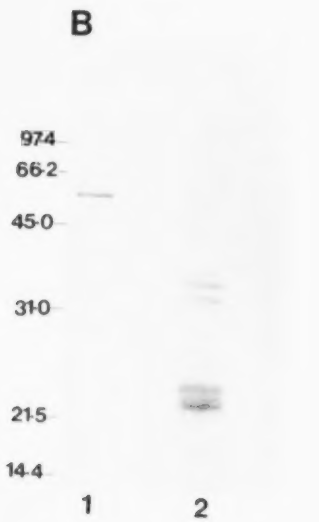

97·4
66·2
45·0
31·0
21·5
14·4
97·4
66·2
45·0
31·0
21·5
14·4
A
B

Figure 9

Western blot analysis of crystalloid protein to examine the specificity of polyclonal antibodies that were raised against the crystalloid protein that had been solubilized initially under (A) non-reducing conditions or (B) reducing conditions (+ME). In each case, lane 1 is a crystalloid protein standard run under non-reducing conditions and lane 2 is a crystalloid protein standard run under reducing conditions (+ME). Numerical values adjacent to the gels are mol wt in kD


A
97·4
66·2
45·0
31·0
21·5
14·4
1
2


B
97·4
66·2
45·0
31·0
21·5
14·4
1
2

respectively. As shown in the figure, antibodies to the non-reduced crystalloid protein showed crossreactivity to only a few of the 30 kD subunit polypeptides and showed no crossreactivity with any of the 20 kD polypeptides. However, antibodies to the reduced crystalloid protein showed crossreactivity to all the subunit polypeptides in the 30 and 20 kD region of the gel. Because of this difference in specificity, the antibodies raised against reduced crystalloid proteins were used for the subsequent studies described in this thesis.

3.5 Determination of the crystalloid protein antibody specificity

3.5.1 Purification of castor bean soluble matrix proteins

In order to determine if the crystalloid protein antibodies obtained in this study crossreacted with other castor bean proteins, soluble matrix proteins from castor bean were first purified on a DEAE-cellulose column. The chromatographic profile yielded three elution peaks as shown in Figure 10. SDS-PAGE analysis of the proteins in each peak revealed that the first peak was the soluble matrix albumins, and the two peaks that followed were the lectins (of which the major proteins were ricin D and ricin CA_1 respectively), that were described by Tully and Beevers (1976). Samples of each class of matrix proteins were boiled in Laemli buffer for 5 minutes and then subjected to SDS-PAGE. After a western transfer of proteins, the proteins were challenged with the crystalloid protein antibodies. Figures 11 and 12 show the gel profiles of the crystalloid proteins, used as a standard, and the matrix proteins, under non-reducing and reducing conditions respectively. The gel profiles after Ponceau staining are shown in (A), and their corresponding immunoblots are shown in (B). In Figure 11, lane 1 represents crystalloid protein, and the 3 peaks lanes 2, 3, and 4 represent the soluble matrix proteins. In Figure 12, lanes 1, 2 and 3 represent soluble matrix proteins and lane 4 represent the crystalloid proteins. As the Figures show, there was

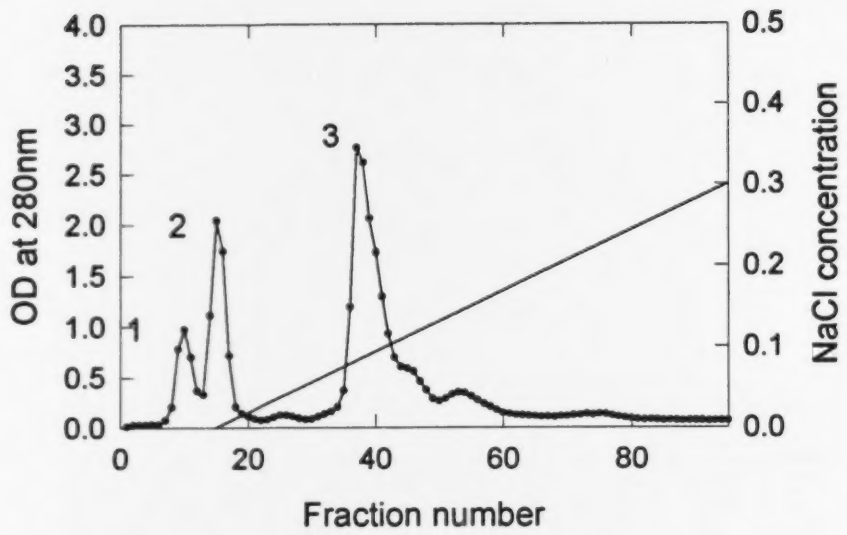


Figure 10

DEAE-cellulose chromatography of soluble matrix fraction from isolated protein bodies. After applying the extract, the column was washed with 5 mM tris-HCl buffer at pH 8.5. The bound proteins were eluted with a linear NaCl gradient. Each fraction was 3 ml.

Figure 11

(A) Ponceau stained SDS-PAGE profiles of non-reduced proteins. (B) Western blot of proteins; (A) after challenge with antibodies raised against reduced crystalloid proteins. Lane 1 is the crystalloid protein standard, lane 2, 3 and 4 represent matrix proteins 1, 2 and 3 respectively following DEAE-cellulose chromatography (see Figure 10). The numerical values adjacent to the gels are mol wt in kD.

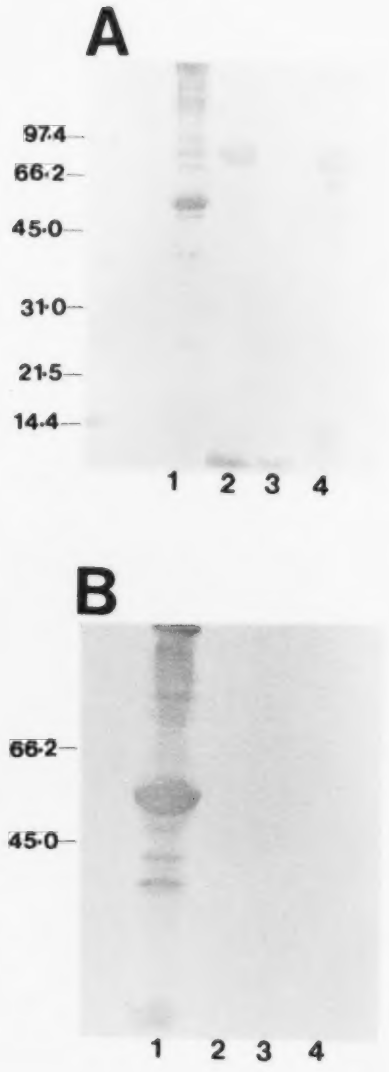
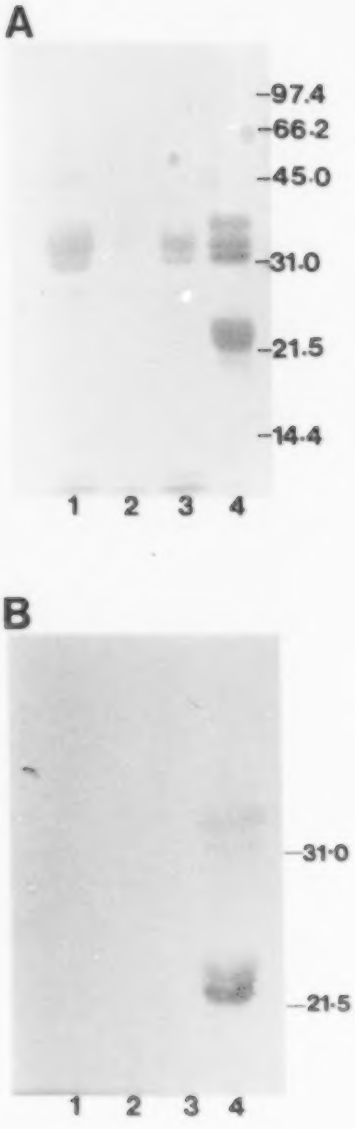

A
97·4
66·2
45·0
31·0
21·5
14·4
1 2 3 4
B
66·2
45·0
1 2 3 4

Figure 12

(A) Ponceau stained SDS-PAGE profiles of reduced proteins. (B) Western blot of proteins; (A) after challenge with antibodies raised against reduced crystalloid proteins. Lane 4 is the crystalloid protein standard, lane 1, 2 and 3 represent matrix proteins 1, 2 and 3 respectively following DEAE-cellulose chromatography (see Figure 10). The numerical values adjacent to the gels are mol wt in kD.


A
97.4
66.2
45.0
31.0
21.5
14.4
1 2 3 4
B
31.0
21.5
1 2 3 4

no crossreactivity between the soluble matrix proteins and the crystalloid protein antibodies.

3.5.2 Determination of immunological relatedness of castor bean storage proteins with storage proteins from other seed types

SDS-PAGE gel profile of insoluble storage proteins extracted from castor bean (*Ricinus communis*), pea (*Pisum sativum*), loblolly pine (*Pinus taeda*), Douglas fir (*Pseudotsuga menziesii*), white spruce (*Picea glauca*), bean (*Phaseolus vulgaris*), and canola (*Brassica napus*) are shown in Figure 13 and Figure 14. In each figure, A shows the SDS-PAGE profiles after coomassie blue staining, and B shows their corresponding western blots, probed with antibodies raised against the reduced castor bean crystalloid protein. It is evident that there was no crossreactivity of castor bean crystalloid antibodies to any storage protein extracted from the species tested.

3.6 Steps involved in storage protein hydrolysis

3.6.1 Gel profiles of insoluble proteins following imbibition, under reducing and non-reducing conditions

To determine changes in insoluble proteins following imbibition, endosperms were taken from mature castor bean seeds, and castor bean seeds 1 to 4 DAI. Insoluble proteins were extracted and subjected to SDS-PAGE under reducing and non-reducing conditions. Figures 15 A and 15 B show coomassie stained gel profiles obtained following SDS-PAGE. As is evident from the figures, the characteristic patterns of castor bean crystalloid proteins, as described by Gifford and Bewley (1983), do not change following imbibition. At each stage, a major protein band was observed in the 50 kD region of the gel under non-reducing, and two polypeptide

Figure 13

SDS-PAGE gel profile of insoluble storage proteins run under non-reducing conditions, after (A) staining with coomassie blue and (B) its corresponding immunoblot. The proteins were extracted from mature seeds of *Phaseolus vulgaris* (a), *Picea glauca* (b), *Pinus taeda* (c), *Pisum sativum* (d), *Pseudotsuga menziesii* (e), *Brassica napus* (f), and *Ricinus communis* (g). The antibodies in the immunoblot were obtained from reduced crystalloid proteins. The numerical values are mol wt in kD.

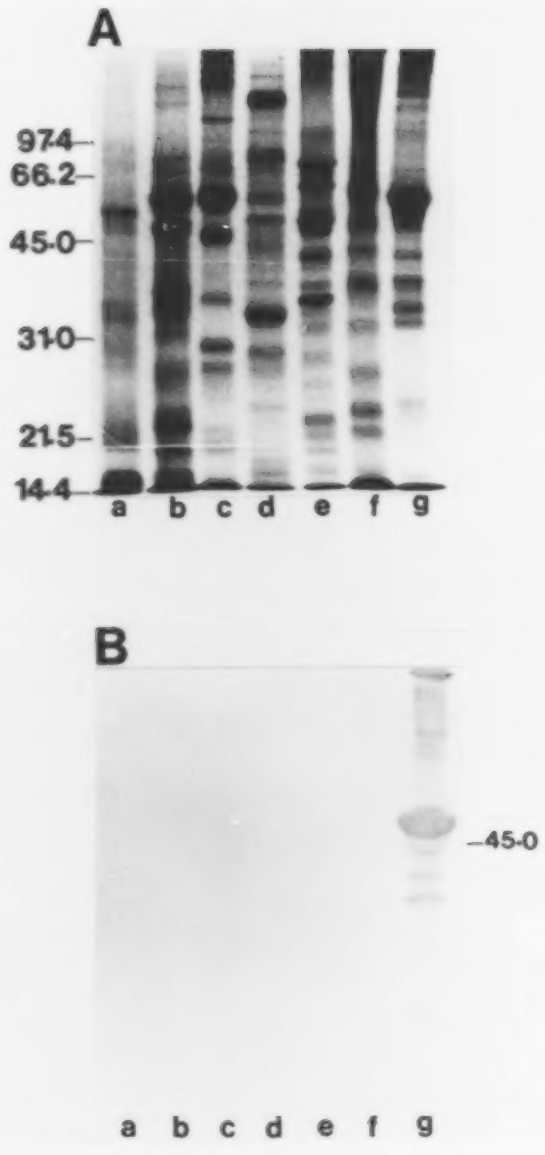

A
97.4
66.2
45.0
31.0
21.5
14.4
a b c d e f g
B
45.0
a b c d e f g

Figure 14

SDS-PAGE gel profile of insoluble storage proteins run under reducing conditions, after (A) staining with coomassie blue and (B) its corresponding immunoblot. The proteins were extracted from mature seeds of *Phaseolus vulgaris* (a), *Picea glauca* (b), *Pinus taeda* (c), *Pisum sativum* (d), *Pseudotsuga menziesii* (e), *Brassica napus* (f), and *Ricinus communis* (g). The antibodies in the immunoblot were obtained from reduced crystalloid proteins. The numerical values are mol wt in kD.

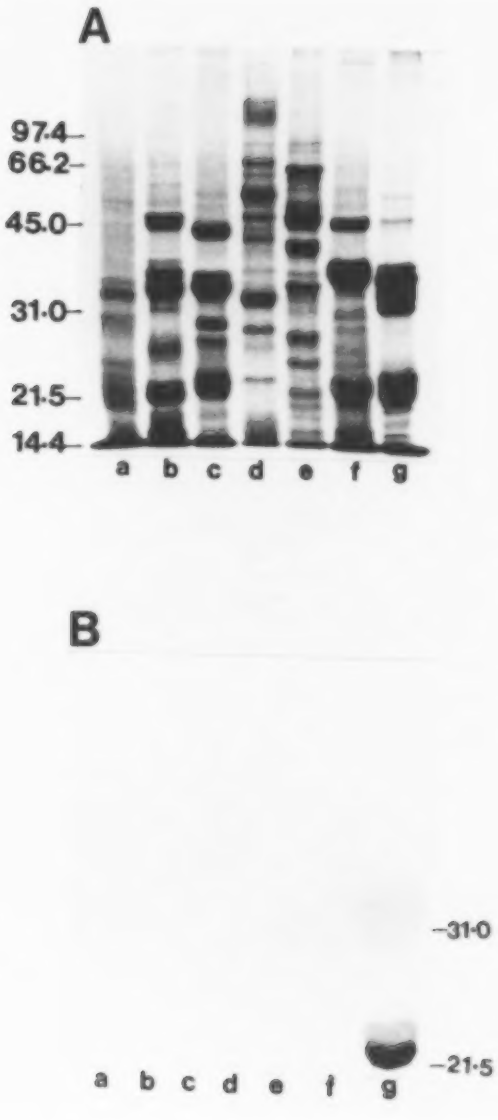
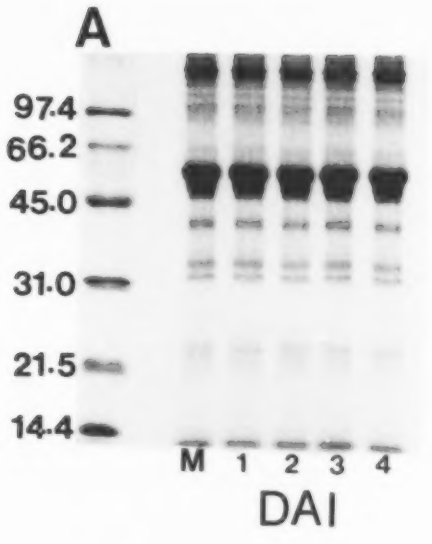
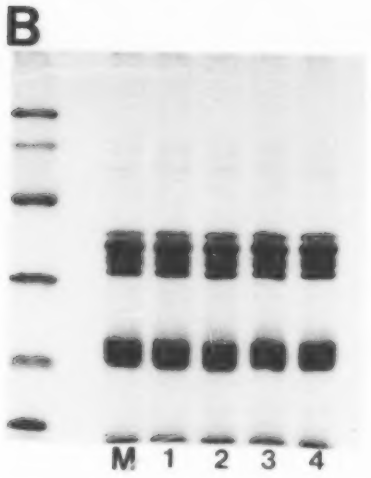

A
97.4
66.2
45.0
31.0
21.5
14.4
a b c d e f g
B
31.0
21.5
a b c d e f g

Figure 15

Coomassie blue stained SDS-PAGE gel profiles of insoluble proteins from castor bean endosperm at 1 to 4 DAI. The profiles were obtained under (A) non-reducing conditions and (B) reducing conditions. Lane M represent proteins extracted from mature seed. The amount of protein loaded on the gel each time was 2 μg. The adjacent numerical values are mol wt in kD.


A
97.4
66.2
45.0
31.0
21.5
14.4
M 1 2 3 4
DAI


B
M 1 2 3 4

groups, one in 30 kD region of the gel and the other in 20 kD region of the gel, were observed under reducing conditions.

3.6.2 ***In vivo*** *analysis of the steps involved in the crystalloid protein hydrolysis*

A previous study by Gifford et al. (1986) showed that the first products of crystalloid protein hydrolysis are soluble proteins. Therefore to study the steps involved in the storage protein hydrolysis, the soluble protein fraction from mature castor bean endosperms and from castor bean endosperms harvested at different stages following seed imbibition (from 6 to 96 hrs), was obtained. These fractions were then subjected to SDS-PAGE followed by immunoblot analysis using antibodies from the reduced crystalloid proteins. The results for soluble fractions run under non-reducing conditions are shown in Figure 16 and for fractions run under reducing conditions in Figure 17. These Figures show that the production of soluble proteins that are recognized by the crystalloid protein antibodies occurs in a set pattern. In the mature seed (M) for example, there is only a little crystalloid protein hydrolysis that occurs. However, by 6 HAI soluble polypeptides begin to appear. Figure 16 shows them to have approximate molecular masses between 40 kD and 50 kD, if the gels are run under non-reducing conditions. When these proteins are separated by SDS-PAGE under reducing conditions, two sets of polypeptides are resolved, one in 30 kD region and the other in 20 kD region of the gel (Figure 17). This pattern hydrolysis continued until 18 HAI. However, after 18 HAI (which corresponds with radicle emergence), the profile pattern changed. At this time the high molecular weight polypeptides disappear and polypeptides with molecular weights in the 40 kD region of the gel began to appear (Figure 16). Following SDS-PAGE under reducing conditions, polypeptides in the 20 kD region of the gel began to appear (Figure 17). These polypeptide patterns continued until 96 HAI.

Figure 16

(A) SDS-PAGE profiles, after coomassie blue staining, of changes in soluble proteins from castor bean endosperms extracted from seeds at different HAI, and (B) their corresponding immunoblots probed with crystalloid protein antibodies. M represents the proteins extracted from mature seed endosperms. Proteins were separated under non-reducing conditions. In all stages, 10 μg protein in 10 μl of Laemmli buffer was applied to the gel. Numerical values to the left of the profiles are mol wt in kD, obtained using Bio-Rad molecular weight standards.

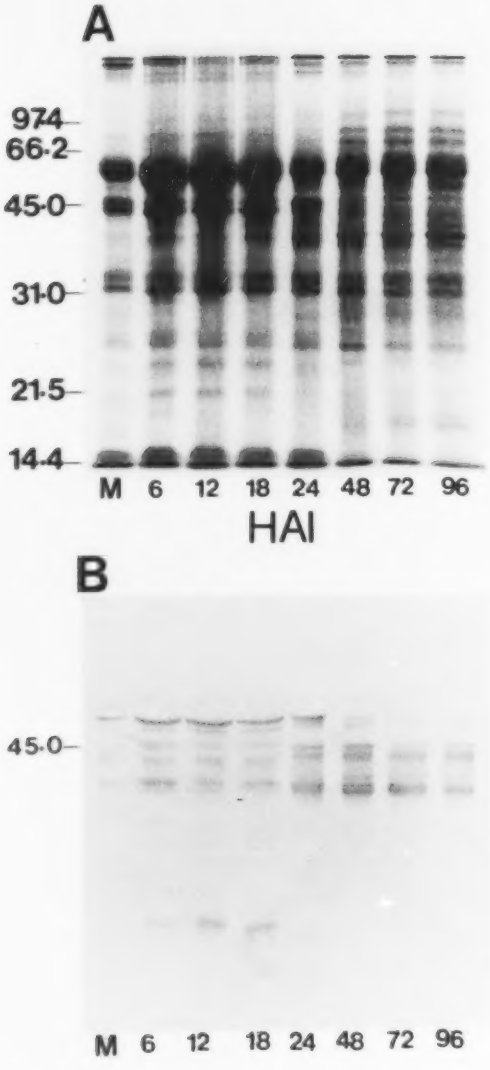
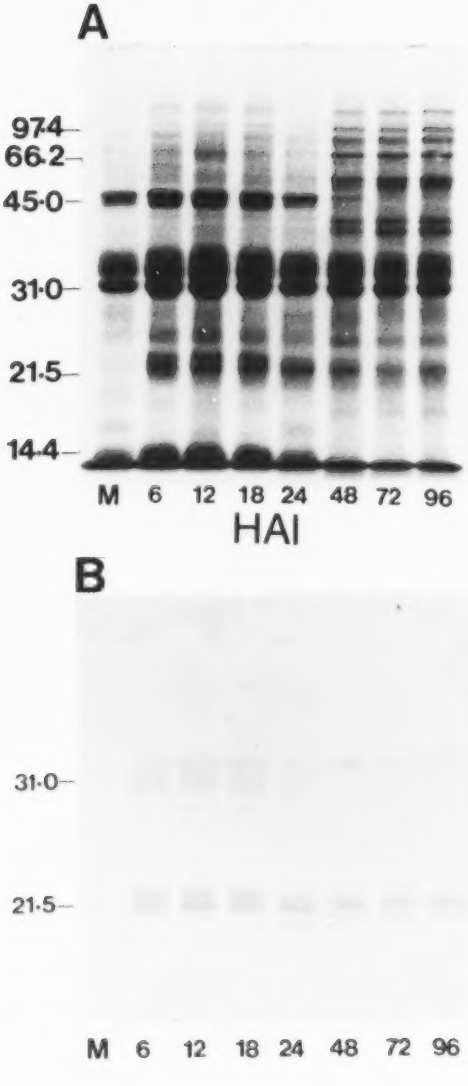

A
97·4
66·2
45·0
31·0
21·5
14·4
M 6 12 18 24 48 72 96
HAI
B
45·0
M 6 12 18 24 48 72 96

Figure 17 (A) SDS-PAGE profiles, after coomassie blue staining, of changes in soluble proteins from castor bean endosperms extracted from seeds at different hours after imbibition (HAI), and (B) is their corresponding immunoblots probed with crystalloid protein antibodies. M represents the proteins extracted from mature seed endosperms. Proteins were separated under reducing conditions. In all stages, 10 μg protein in 10 μl of Laemmli buffer was applied to the gel. Numerical values to the left of the profiles are mol wt in kD, obtained using Bio-Rad molecular weight standards.


A
97·4
66·2
45·0
31·0
21·5
14·4
M 6 12 18 24 48 72 96
HAI
B
31·0
21·5
M 6 12 18 24 48 72 96

3.6.3 Effect of cycloheximide on in-vivo storage protein hydrolysis

In order to find out if the observed hydrolysis product patterns were due to preformed enzymes or due to enzymes that are synthesized *de novo*, castor bean seeds were imbibed in different concentrations of cycloheximide (0 μM, 10 μM and 100 μM), and soluble proteins were obtained from these seeds and analyzed. A comparison between Figures 16 and 17 and Figures 18 and 19 shows that very similar profiles were obtained when seeds were imbibed in 0 μM or in 10 μM cycloheximide. In each figure, (A) represents the gel profiles after staining with coomassie blue and (B) represents the corresponding immunoblots after probing with the crystalloid protein antibodies. However, when the seeds were imbibed in 100 μM, a different result was obtained. The gel profiles are shown in Figures 20 and 21. In this case only polypeptides in the 40 kD region of the gels run under non-reducing conditions were obtained. These were resolved as polypeptides in the 20 kD region of the gel when reducing conditions were used.

3.7 *In-vitro* analysis of crystalloid protein hydrolysis

To minimize the presence of products of storage protein hydrolysis in the enzyme extract, mature seeds were used for the enzyme extraction. Using non-labeled crystalloid protein substrate in an enzyme reaction mixture as described in the materials and methods, an *in-vitro* analysis was carried out over 48 hours. After each time period of the reaction, the soluble reaction products were boiled in Laemmli buffer and subjected to SDS-PAGE followed by immunoblotting. Figures 22 and 23 show the analysis of the reaction products carried out under non-reducing and reducing conditions respectively. In both figures, (A) shows the protein gel profiles after staining with coomassie blue, and (B) shows the corresponding immunoblots after probing with crystalloid protein antibodies. As the figures show, the products of

Figure 18

(A) SDS-PAGE profiles, after coomassie blue staining, of changes in soluble proteins from castor bean endosperms that had been imbibed in 10 μM cycloheximide. The proteins were extracted from seeds at different hours after imbibition (HAI), and (B) their corresponding immunoblots probed with crystalloid protein antibodies. M represents the proteins extracted from mature seed endosperms. Proteins were separated under non-reducing conditions. In all stages, 10 μg protein in 10 μl of Laemmli buffer was applied to the gel. Numerical values to the left of the profiles are mol wt in kD, obtained using Bio-Rad molecular weight standards.

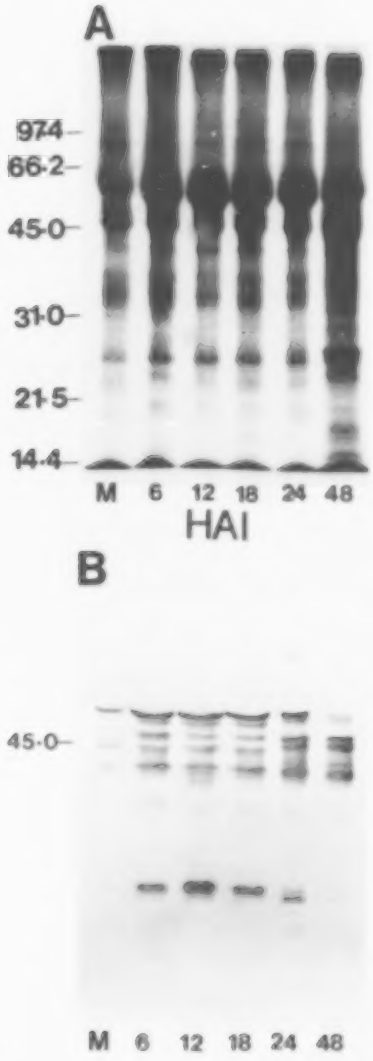
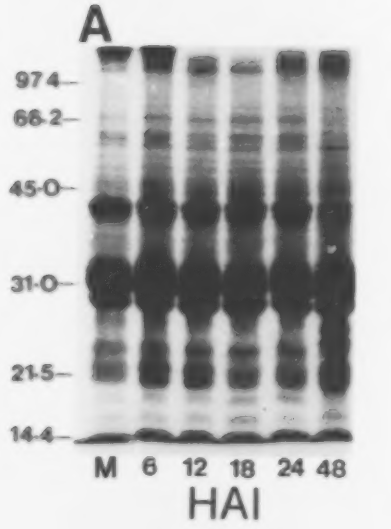

A
97.4
66.2
45.0
31.0
21.5
14.4
M 6 12 18 24 48
HAI
B
45.0
M 6 12 18 24 48

Figure 19

(A) SDS-PAGE profiles, after coomassie blue staining, of changes in soluble proteins from castor bean endosperms that had been imbibed in 10 μM cycloheximide. The proteins were extracted from seeds at different hours after imbibition (HAI), and (B) their corresponding immunoblots probed with crystalloid protein antibodies. M represents the proteins extracted from mature seed endosperms. Proteins were separated under reducing conditions. In all stages, 10 μg protein in 10 μl of Laemmli buffer was applied to the gel. Numerical values to the left of the profiles are mol wt in kD, obtained using Bio-Rad molecular weight standards.


A
97·4
66·2
45·0
31·0
21·5
14·4
M 6 12 18 24 48
HAI

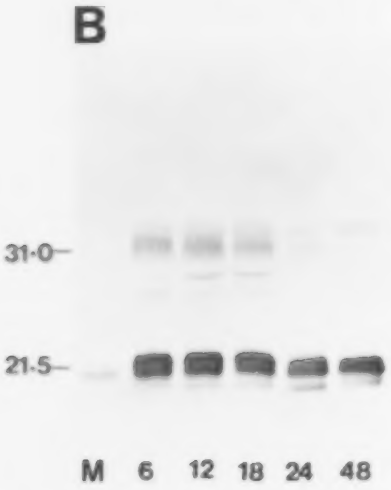

B
31·0
21·5
M 6 12 18 24 48

Figure 20

(A) SDS-PAGE profiles, after coomassie blue staining, of changes in soluble proteins from castor bean endosperms that had been imbibed in 100 μM cycloheximide. The proteins were extracted from seeds at different hours after imbibition (HAI), and (B) their corresponding immunoblots probed with crystalloid protein antibodies. M represents the proteins extracted from mature seed endosperms. Proteins were separated under non-reducing conditions. In all stages, 10 μg protein in 10 μl of Laemmli buffer was applied to the gel. Numerical values to the left of the profiles are mol wt in kD, obtained using Bio-Rad molecular weight standards.

A
97.4
66.2
45.0
31.0
21.5
14.4
M 6 12 18 24 48
HAI
B
45.0
M 6 12 18 24 48

Figure 21

(A) SDS-PAGE profiles, after coomassie blue staining, of changes in soluble proteins from castor bean endosperms that had been imbibed in 100 μM cycloheximide. The proteins were extracted from seeds at different hours after imbibition (HAI), and (B) their corresponding immunoblots probed with crystalloid protein antibodies. M represents the proteins extracted from mature seed endosperms. Proteins were separated under reducing conditions. In all stages, 10 μg protein in 10 μl of Laemmli buffer was applied to the gel. Numerical values to the left of the profiles are mol wt in kD, obtained using Bio-Rad molecular weight standards.

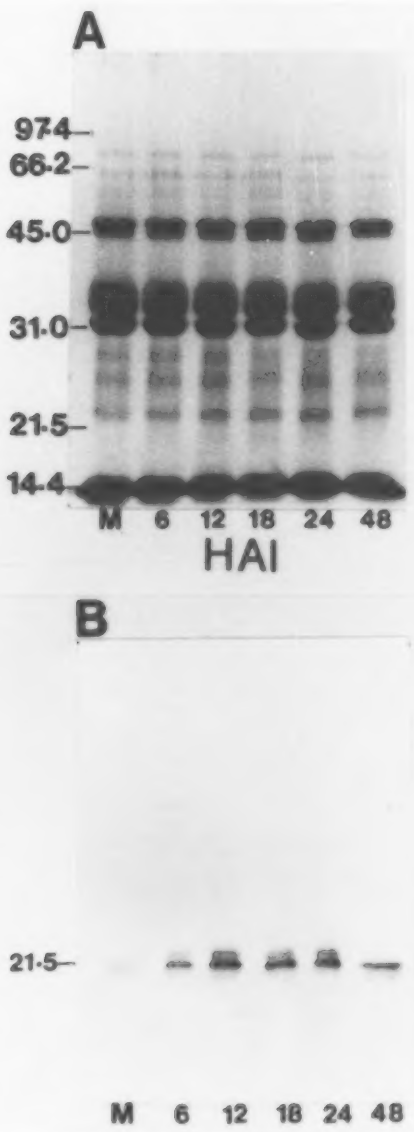

A
97·4
66·2
45·0
31·0
21·5
14·4
M 6 12 18 24 48
HAI
B
21·5
M 6 12 18 24 48

Figure 22

In-vitro analysis of crystalloid protein hydrolysis. (A) SDS-PAGE profiles, after coomassie blue staining, of proteins taken from soluble reaction products and (B) their corresponding immunoblots probed with crystalloid protein antibodies. The reactions were carried out at the following intervals: 0, 6, 12, 18, 24, and 48 hrs. Proteins were separated under non-reducing conditions. Numerical values to the left of the profiles are mol wt in kD, obtained using Bio-Rad molecular weight standards.

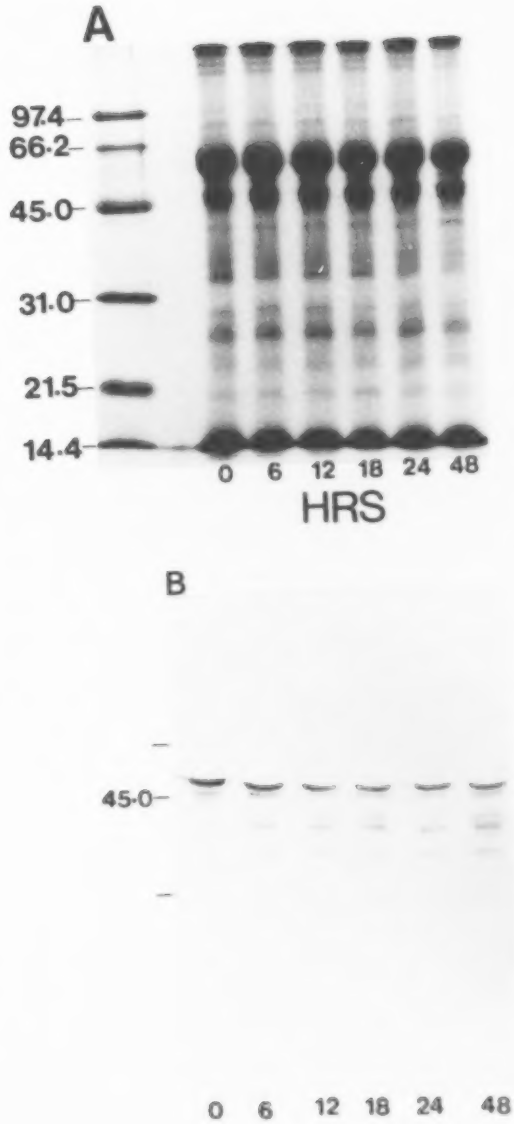
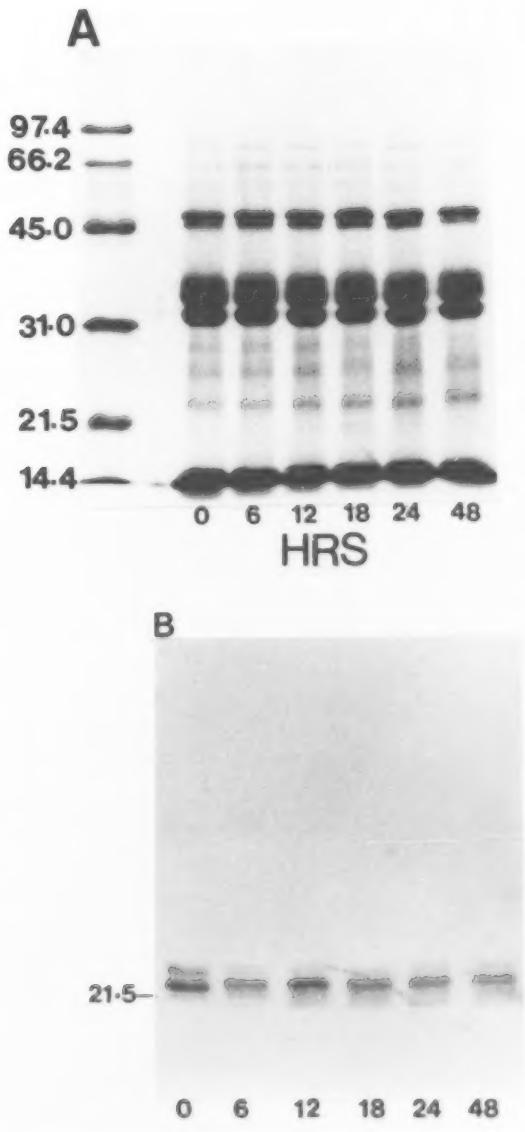

A
97.4
66.2
45.0
31.0
21.5
14.4
0 6 12 18 24 48
HRS
B
45.0
0 6 12 18 24 48

Figure 23

In-vitro analysis of crystalloid protein hydrolysis. (A) SDS-PAGE profiles, after coomassie blue staining, of proteins taken from soluble reaction products and (B) their corresponding immunoblots probed with crystalloid protein antibodies. The reactions were carried out at the following intervals: 0, 6, 12, 18, 24, and 48 hrs. Proteins were separated under reducing conditions. Numerical values to the left of the profiles are mol wt in kD, obtained using Bio-Rad molecular weight standards.


A
97.4
66.2
45.0
31.0
21.5
14.4
0 6 12 18 24 48
HRS
B
21.5
0 6 12 18 24 48

hydrolysis which appeared in the 40 kD region of the gel under non-reducing conditions and in the 20 kD region of the gel under reducing conditions were first observed in the mature seed. Similar patterns of protein bands were observed over the 48 hr experimental period. These results are similar to those obtained when the seeds were imbibed in 100 μM cycloheximide.

3.8 Immunoprecipitation of [^{35}S] methionine labeled crystalloid protein reaction products

After in-vivo labeling of crystalloid protein with [^{35}S]-methionine for 6 hrs, extraction of the insoluble fraction and the subsequent immunoprecipitation was performed as described in "Materials and Methods". At the beginning of the experiment there were high levels radiolabeled proteins, as determined by high radioactive counts in the reaction product samples. However, high enough radioactive counts to show prominent bands after SDS-PAGE and fluorography could not be obtained from the *staphylococcus* cells at the end of the immunoprecipitation (data not shown).

IV. Discussion

4.1 Some observations of the structure of 11S seed storage proteins

The holoprotein of the 11S class of seed storage proteins, the seed globulins, is made up of six identical subunits of approximate molecular masses of 50 kD. Each subunit comprises two polypeptides, an acidic one with approximate molecular mass of 30 kD and a basic one with approximate molecular mass of 20 kD. According to Plietz et al., (1987) who investigated amino acid sequences of 11S globulins in *Vicia faba* and *Pisum sativum* by computer analysis, 11S globulin subunits are arranged with the strongly hydrophilic α-chain (the 30 kD acidic polypeptide) at the surface of the globulin molecule and the more hydrophobic β-chain (the 20 kD basic polypeptide) in the central part of the molecule. The structural arrangement of the 11S seed storage proteins is likely to be an evolutionary adaptation to provide a high packing density of these storage proteins within the protein body. If the castor bean 11S proteins (the crystalloid proteins) have a similar structural arrangement, then it would explain the peculiar results that were obtained in this thesis while determining the specificity of the antibodies to the native crystalloid protein. These results showed that antibodies raised against crystalloid proteins that had been solubilized under non-reducing conditions (-ME), showed crossreactivity to only a few of the 30 kD subunit polypeptides and showed no crossreactivity at all to the 20 kD subunit polypeptides.

On the other hand, antibodies raised to the reduced crystalloid proteins showed crossreactivity to all subunit polypeptides in the 30 and 20 kD regions of the gel. If the castor bean crystalloid acidic subunit polypeptides (30 kD) are situated on the surface of the holoprotein, and surround the basic polypeptides (20 kD), then antibodies raised to the non-reduced crystalloid protein, would only recognize those antigenic sites on the surface of the molecule, the 30 kD peptides. The antigenic sites on the polypeptides that are in centre of the molecule, the 20 kD polypeptide, which are sheltered from the outside, would not be recognized. However, antibodies raised to reduced crystalloid proteins, where all the antigenic sites on both the acidic and basic polypeptides were exposed, since they are now both free in solution, would show the observed antibody crossreactivity to all the subunit polypeptides..

4.1.1 Immunological homology of castor bean storage proteins with storage proteins from other seed types

Unlike previous studies of seed storage proteins that reported immunological homology between 11S proteins of gymnosperms and 11S proteins of angiosperms (Allona et al.,1992, Misra and Green, 1994), this study showed no immunological relatedness between 11S proteins that were tested and the 11S proteins (the crystalloid proteins) from castor bean. The discrepancy in these findings is probably due to the different methods used in raising the antibodies. Antibodies used in this thesis were raised against the reduced castor bean crystalloid protein, whereas Allona et al., (1992) used antibodies raised against the 21 kd polypeptide from *Pinus pinaster* and

Misra and Green (1994) used antibodies from non-reduced crystalloid protein from white spruce. The immunological homology between 11S globulins reported by Misra and Green was based on an immunoblot analysis of proteins from various angiosperm seeds, including castor bean. It is not unreasonable to assume that, if these proteins were truly immunologically homologous, antibodies to the white spruce proteins should detect similar proteins from castor bean and vice versa. The immunological homology claimed by Misra and Green is doubtful since the castor bean antibodies used in this thesis showed no crossreactivity with white spruce 11S proteins.

4.2 Storage protein hydrolysis

4.2.1 Changes in hydrolytic enzyme activity following seed imbibition

The activity of enzymes capable of hydrolyzing the crystalloid proteins was high in cell free extracts of the mature seed endosperm. Following seed imbibition, the enzyme activity remained high and continued at this level throughout germination. However, following germination, the activity of these enzymes increased, reaching a maximum two days after radical emergence. This increased activity is likely due to a set of enzymes that are synthesized *de novo*, since there was no increase in enzyme activity when the seeds were imbibed in 100 μM cycloheximide, a protein synthesis inhibitor (Alberts et al., 1994). In contrast, the activity in cell free extracts from mature seeds or seeds taken during germination could be due to an existing enzyme or

set of enzymes that were synthesized during seed development, and conserved in the seed during maturation since this activity was not affected by cycloheximide. This has been previously shown to be the case in french bean seeds (Yomo and Srinivasan, 1973), pumpkin (Hara and Matsubara, 1980) and castor bean (Gifford et al., 1986). However, as the following discussion will show, this latter conclusion is likely too simplistic.

4.2.2 Immunological crossreactivity of crystalloid protein antibodies with other proteins from castor bean endosperm

Since the hydrolysis of castor bean crystalloid proteins yields soluble products (Gifford et al., 1986), it was important to determine that the crystalloid protein antibodies did not crossreact with other castor bean soluble proteins. Immunoblot analysis showed no crossreactivity between crystalloid protein antibodies with the castor bean matrix proteins. This result suggests that these antibodies were very specific to crystalloid proteins.

4.2.3 Steps involved in crystalloid protein hydrolysis

Because the crystalloid protein hydrolysis products were soluble, we were able to detect these products using antibodies without interference from the unhydrolyzed substrate. This fact provided a unique opportunity to follow the hydrolysis of the crystalloid proteins in the imbibed seeds. In this way we could determine if the nature of this hydrolysis changed during germination and early seedling growth. While this approach is not new, data from other studies using a similar approach have been

difficult to interpret since in these cases both the substrate and products were soluble and both were detected in the same extract, e.g. Boylan and Sussex (1987). The results presented in this thesis show that there were small amounts of crystalloid protein breakdown products in cell-free extracts of mature castor bean seed endosperms. These hydrolysis products appear to be homodimers, with molecular masses of approximately 40 kD. By 6 HAI, however, a new protein pattern was obtained. This was due to the production of heterodimers comprising peptides with approximate molecular masses of 20 and 30 kD respectively. This pattern continued until 18 HAI when a third set of hydrolysis products appeared. These peptides were homodimers with molecular masses of approximately 40 kD. This final pattern continued until the seeds began to degenerate due to senescence. These data suggest that crystalloid protein hydrolysis occurred in stages involving at least 3 different sets of enzymes. This is illustrated in Figure 24. In this model, the first class of enzymes would be synthesized during seed development and conserved in the mature seed. Although their potential activity is high in the mature seed, as shown by hydrolysis of [^{3}H]-leucine labeled crystalloid protein, this set of enzymes appears to have low activity *in vivo*. This is because there is only a small pool of crystalloid protein hydrolysis products produced in the mature seed extracts *in vivo*. This activity appears to increase *in vivo* following imbibition since significant amounts of 40 kD hydrolysis products are produced in the seeds imbibed in the presence of 100 μM cycloheximide. Also these enzymes are likely to have long half-lives, since 40 kD homodimers were still observed in extracts of seeds that had been imbibed for up to 48 hrs in the presence of cycloheximide. A similar hydrolysis of storage proteins during early germination that was insensitive to cycloheximide has been reported previously (Yomo and Srinivasan, 1973, Hara and Matsubara, 1980 and Gifford et al.,1986). The

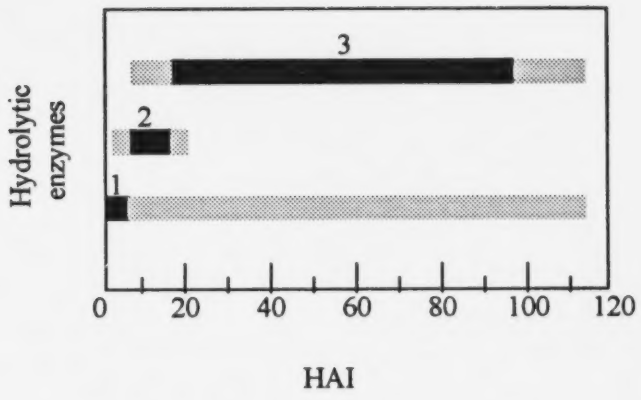


Figure 24

A model of enzymes that hydrolyze crystalloid proteins in castor bean endosperm. (1) conserved enzyme that is responsible for the initial hydrolysis observed in the mature seed, (2) enzyme that is synthesized *de novo* by preformed mRNA and (3) enzyme that is synthesized*de novo* by newly transcribed mRNAs.

homodimers produced by these enzymes are probably intermediates required to produce amino acids that are needed to support germination and the synthesis of new hydrolytic enzymes following the completion of the germination process. In relation to this, Gifford et al. (1986) showed that there was a significant increase in the free amino acid pool in the endosperm of castor bean prior to radicle emergence. A similar role can be attributed to the second class of enzymes. These are responsible for the production of the protein pattern observed by 6 HAI, and are likely the result of translation of mRNAs that are formed during seed maturation and then conserved in the mature seed. Evidence for this comes from the cycloheximide study which showed that these peptides were absent when the seeds were imbibed in the presence of 100 μM cycloheximide. A similar argument can be made for the third class of enzymes, responsible for the production of the protein pattern obtained after 18 HAI in that their synthesis is due to mRNAs that are newly transcribed *de novo* and then translated. In this model, the third class of enzymes would be responsible for the rapid storage protein hydrolysis that occurs following radicle emergence. The hydrolysis products would then be used to support seedling growth until autotrophy is achieved. Interestingly, the peptides produced by the third class of proteases are similar to those produced by the first class of proteases present in the mature seed.

4.3 Biochemical characterization of enymes that are involved in storage protein hydrolysis

As a first approach to a biochemical characterization of crystalloid protein endopeptidases in castor bean, the enzymes representing the third class of storage protein hydrolases were studied. This was because they showed highest activity in cell-free extracts. Many previous studies of proteolytic enzymes in germinating seeds have used chromogenic substrates (Cornel and Plaxton, 1994, Tanaka et al., 1993, Endo et al., 1987, Mitsuhashi and Minamikawa, 1989, Salmia, 1981, de Moraes et al., 1994). Hydrolysis of chromogenic substrates, however, does not indicate the exact steps involved in the hydrolysis of the native storage proteins. To address this problem, in this study the native storage protein (crystalloid protein) was used as a substrate. The assay system relied on the observation that while the native crystalloid protein was insoluble in dilute buffers, its hydrolytic products were buffer soluble (Gifford et al., 1986). Using [^{3}H]-leucine and [^{35}S]-methionine labeled crystalloid proteins as substrate, it was possible to measure the rate of proteolytic enzyme activity by determining the amount of radioactivity generated in soluble fractions of the reaction products.

The enzymes that hydrolyze [^{3}H]-leucine labeled crystalloid protein exhibited an acidic pH optimum of 5. Similar acidic pH optima for enzymes shown to initiate the proteolysis of storage proteins in various seeds have been previously shown (Baumgartner and Chrispeels, 1977, Boylan and Sussex, 1987, Mitsuhashi and Minamikawa, 1989, Qi et al., 1992. The pH optimum for the other class of enzymes, at earlier stages was not determined in this study.

4.4 Future research

With the solid foundation, on the steps and the enzyme classes involved in castor bean storage protein hydrolysis, established in this study, the stage is now set for their purification and biochemical characterization. The cloning of the genes for endopeptidases representing each enzyme class would give us greater insight into their regulation. For example cDNA clones of genes for each of the three endopeptidase classes could be used to probe mRNAs obtained following seed imbition. These would confirm the presence of mRNAs for class 2 and 3 enzymes; mRNAs for class 1 enzymes should not be present. Additionally, the mRNAs for class 2 enzymes should decrease after 18 HAI whereas mRNAs for class 3 enzymes should be observed until seed senescence.

In summary, data presented in this thesis have shown that castor bean crystalloid protein hydrolysis occurs in three stages and involves three classes of proteases. In addition, an assay system has been developed that uses the crystalloid protein as substrate. This assay will allow the purification and biochemical characterization of these enzymes to be completed.

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